Estradiol-induced expression of Na\(^{+}\)-K\(^{+}\)-ATPase catalytic isoforms in rat arteries: gender differences in activity mediated by nitric oxide donors

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Palacios, Javier, Elisa T. Marusic, Nandy C. Lopez, Magdalena Gonzalez, and Luis Michea. Estradiol-induced expression of Na\(^{+}\)-K\(^{+}\)-ATPase catalytic isoforms in rat arteries: gender differences in activity mediated by nitric oxide donors. *Am J Physiol Heart Circ Physiol* 286: H1793–H1800, 2004. First published January 2, 2004; 10.1152/ajpheart.00990.2003.—We tested the hypothesis that previously demonstrated gender differences in ACh-induced vascular relaxation could involve diverse Na\(^{+}\)-K\(^{+}\)-ATPase functions. We determined Na\(^{+}\)-K\(^{+}\)-ATPase by measuring arterial ouabain-sensitive \(^{86}\)Rb uptake in response to ACh. We found a significant increase of Na\(^{+}\) pump activity only in aortic rings from female rats (control 206 ± 11 vs. 367 ± 29 nmol \(^{86}\)Rb/K/min·g wt tissue\(^{-1}\); \(P<0.01\)). Ovariectomy eliminated sex differences in Na\(^{+}\)-K\(^{+}\)-ATPase function, and chronic in vivo hormone replacement with 17\(^{\beta}\)-estradiol restored the ACh effect on Na\(^{+}\)-K\(^{+}\)-ATPase. Because ACh acts by enhancing production of NO, we examined whether the NO donor sodium nitroprusside (SNP) mimics the action of ACh on Na\(^{+}\)-K\(^{+}\)-ATPase activity. SNP increased ouabain-sensitive \(^{86}\)Rb uptake in denuded female arteries (control 123 ± 7 vs. 197 ± 12 nmol \(^{86}\)Rb/K/min·g wt tissue\(^{-1}\); \(P<0.05\)). Methylene blue (an inhibitor of guanylate cyclase) and KT-5823 (a cGMP-dependent kinase inhibitor) blocked the stimulatory action of SNP. Exposure of female thoracic aorta to the Na\(^{+}\)/K\(^{+}\) pump inhibitor ouabain significantly decreased SNP-induced and ACh-mediated relaxation of aortic rings. At the molecular level, Western blot analysis of arterial tissue revealed significant gender differences in the relative abundance of catalytic isoforms of Na\(^{+}\)-K\(^{+}\)-ATPase. Female-derived aortas exhibited a greater proportion of \(\alpha_2\)-isoform (44%) compared with male-derived aortas. Furthermore, estradiol upregulated the expression of \(\alpha_2\) mRNA in male arterial explants. Our results demonstrate that enhancement of ACh-induced relaxation observed in female rats may be in part explained by 1) NO-dependent increased Na\(^{+}\)-K\(^{+}\)-ATPase activity in female vascular tissue and 2) greater abundance of Na\(^{+}\)-K\(^{+}\)-ATPase \(\alpha_2\)-isoform in females.

Acetylcholine; sodium nitroprusside; ouabain; vascular relaxation; rat aorta

Na\(^{+}\)-K\(^{+}\)-ATPase is responsible for the electrochemical gradient of sodium and potassium ions. Therefore, it plays an important role in the regulation of ionic homeostasis in tissues and cells. The enzyme is composed of a catalytic \(\alpha\)-subunit and a \(\beta\)-subunit that is essential for enzyme maturation (7, 37, 38, 66). In vascular smooth muscle cells, Na\(^{+}\)-K\(^{+}\)-ATPase plays a major role in the regulation of vascular tone (8, 14, 66). An increase in Na\(^{+}\)-K\(^{+}\)-ATPase activity leads to hyperpolarization and relaxation of smooth muscle, whereas its inhibition by ouabainlike compounds induces the opposite effect (1, 9, 10, 50).

A variety of earlier studies demonstrated the existence of significant male-female differences in vascular reactivity (55, 58, 68). ACh-induced relaxation is greater in female than male rats (56, 71), and this difference is endothelium dependent (40, 53, 71). Several studies postulated that estrogens play an important role in modulating vascular responses to vasodilators (12) and that this effect is mediated by endothelium. NO plays an important role in maintaining the cardiovascular system in a state of constant active vasodilation (43). More recent studies clearly established that estrogens exert effects on endothelial function and vascular smooth muscle contractile force, which contribute to sex differences in vascular tone (26, 32, 39). Also, estrogens upregulate endothelial nitric oxide synthase via a receptor-mediated system (23, 25, 63).

NO donors induce vascular smooth muscle relaxation mainly through the activation of soluble guanylate cyclase and the subsequent increase in cGMP levels (57). Although cGMP-independent mechanisms have been reported (71), the relaxation induced by this cyclic nucleotide may involve a decrease in intracellular Ca\(^{2+}\) concentration (6, 17, 33) and different potassium channels (4, 45, 70, 72) to the closure of L-type Ca\(^{2+}\) channels (54) and/or activation of Na\(^{+}\)-K\(^{+}\)-ATPase (50, 65). It is likely that the importance of a given mechanism may markedly differ not only in different vascular beds (16) but also with gender. So far, little is known about possible gender differences in vascular Na\(^{+}\)-K\(^{+}\)-ATPase activity.

The purpose of the present study was to determine whether the specific male-female differences in smooth muscle relaxation are associated with changes in the function of the Na\(^{+}\) pump. We studied the effect of ACh and of a NO donor on the Na\(^{+}\) pump in arterial vessels of male, female, and ovariectomized rats with or without hormone replacement therapy. In addition, protein abundance and expression of the catalytic isoforms of Na\(^{+}\)-K\(^{+}\)-ATPase were measured in male and female arteries.

METHODS

Animals. Sexually mature male and female Sprague-Dawley rats (13 wk of age) were used. All rats were housed in groups of two or three in a temperature-controlled, light-cycled (8:00 AM–8:00 PM) room with ad libitum access to water and standard rat chow (Champion). The state of the estrous cycle was determined by vaginal smear, and females in the estrous state were used. All procedures involving the use of animals were approved by the Institutional Animal Care and

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Use Committee. In some experiments, female rats were divided into three groups: control, ovariectomized (OVX), and ovariectomized plus estradiol (OVX+E2). Rats were anesthetized with ether, and the ovaries were ligated and then removed. One week after the surgery, OVX rats were divided into two groups. One group received 17β-estradiol benzoate (20 µg/kg, every 48 h) in vegetal oil for 4 wk by subcutaneous injection, and the other (control) group received vehicle

Isolation of aortic rings. The thoracic aorta was quickly excised and placed in cold (4°C) physiological Krebs-Ringer bicarbonate (KRB) buffer containing (in mM) 4.2 KCl, 1.19 KH2PO4, 120 NaCl, 25 Na2HCO3, 1.2 MgSO4, and 5 glucose (pH 7.4). Rings (3–5 mm and 2–4 mg) were prepared after connective tissue was dissected from the aorta, taking special care to avoid endothelial damage. In some experiments, endothelium-denuded aortic rings were prepared by inserting a stainless steel wire into the lumen and gently rolling the ring on a filter paper soaked in KRB. Aortic rings were equilibrated for 45 min at 37°C in separate vials with 2 ml of KRB in a water-saturated atmosphere containing 95% O2-5% CO2 (Dubnoff incubator). After 45 min of incubation in KRB, tissue samples were used for transport experiments.

Na+ pump activity; 86Rb/K uptake in aortic rings. Na+-K+-ATPase activity was measured by ouabain-sensitive 86Rb/K uptake in aortic rings according to Miecha et al. (41). Briefly, the aortic rings were equilibrated for 45 min in KRB (37°C) in the presence of ouabain (10−3 M) when indicated. Finally, triplicate tissue samples were incubated in 2 ml of KRB containing 86Rb (0.1 mCi/ml) in the presence or absence of ouabain for 20 min, as described previously (11). Transferring the aortic rings into iced KRB stopped the reaction; the tissue was then quickly washed in cold buffer and gently blotted. Radioactivity of the samples was determined by Cerenkov radiation in a liquid scintillation counter in the presence of 0.1% Tween 20 (4 ml). The ouabain-sensitive component of the 86Rb uptake, which is known to be an index of Na+-K+-ATPase activity, was calculated by subtracting ouabain-insensitive 86Rb uptake from the total 86Rb uptake. The results are expressed as nanomoles of 86Rb/K per minute per gram of tissue.

Vascular reactivity experiments. Thoracic aortas were removed and placed in cold (4°C) physiological KRB buffer. The aorta was cleaned of all adipose and connective tissue, and the mithrochondrial region was cut into rings (each 3 mm long). Extreme care was taken during preparation of the rings to avoid stretching the tissue. In each experiment, two adjacent aortic rings were studied from the same animal in paired fashion. The method of Stallone et al. (61) was followed for isometric tension measurement. Briefly, the rings were mounted on two 25-gauge stainless steel wires; the lower one was attached to a stationary glass rod and the upper one was attached to a force-displacement transducer (Grass FT-03C). The transducer was connected to a Grass polygraph (model 7) for a continuous record of blood vessel tension. After the equilibration period, the aortic rings were stabilized by two successive near-maximal contractions with KCl (60 mM). Endothelium-independent relaxations were studied by measuring sodium nitroprusside (SNP)-induced changes in tension (SNP 10−6–10−3 M) of phenylephrine (PE)-contracted (PE 10−6 M) denuded thoracic aortic rings isolated from male and female rats in the presence of ouabain (10−3 M). ACh-dependent relaxation was also measured in intact precontracted aortic rings (PE 10−6 M) of male and female rats in the presence and absence of 10−3 M ouabain.

Arterial explants. Mesenteric arteries and aorta were removed from normal male rats (170–200 g) and cleaned of blood and connective tissue in ice-cold KRB. Mesenteric branches or aortic rings were placed in DMEM high-glucose medium (GIBCO BRL) supplemented with 1% glutamine (US Biological), 1% penicillin-streptomycin solution (10,000 U/ml penicillin, 10 mg/ml streptomycin) and 1% MEM nonessential amino acids solution (GIBCO BRL) according to Jiang et al. (28). Arterial explants were incubated for 24 h in a 5% CO2 atmosphere at 37°C with various concentrations of estradiol (10−7–10−11 M) or vehicle (control). After the incubation period, RNA was extracted and used for semiquantitative RT-PCR experiments to estimate α2 mRNA abundance.

RNA isolation and reverse transcription. Total RNA was isolated from tissue explants by using an RNA extraction reagent TRizol (Invitrogen-Life Technologies) method per the specifications of the manufacturer. Total RNA (0.5 µg) was reverse transcribed with the reverse transcription system (Promega). To confirm that the reverse transcription reaction was unsaturated, PCR amplification was performed with cDNA synthesized from increasing amounts of RNA as a template (0.3–1 µg).

Semiquantitative RT-PCR. PCR was performed in a DNA thermal cycler (Mastercycler Personal, Eppendorf). Primers were used to amplify Na+-K+-ATPase α2-subunit were designed according to the sequence obtained from GenBank (Accession no. D90049). The sense primer (GCTGTTAGCCGAAAATCTGC) and the antisense primer (TGTCCTCTGGGTCTTCTA) amplified a 236-bp product. The sense primer (CAGGACCACTTGGAAAGTC) and the antisense primer (GCTATGGAGCATGGAATTACCG) were used for PCR amplification of the 18S ribosomal RNA, which served as an internal control. PCR conditions were 94°C, 30 s; 60°C, 30 s; and 72°C, 30 s for 25 cycles. 18S ribosomal RNA primers for PCR were included in each reaction in cycle 20 (0.5 µM each). Preliminary experiments demonstrated that α2 and 18S PCR reactions were in the exponential amplification phase.

Membrane preparation and Western blot analysis. Arterial smooth muscle actin (SMA) and α1 and α2 protein levels were measured by Western blot according to Miecha et al. (42). Briefly, total protein homogenate and crude membrane fractions were prepared to minimize the potential selective enrichment of different proteins during the purification procedure. Thoracic aortas from four rats were homogenized by a Polytron (PTA 10–33, Kinematica) in ice-cold buffer containing 50 mM Tris-HCl, pH 7.4, 50 mM β-mercaptoethanol, and one mitintablet of Complete Protease Inhibitor Cocktail (Roche) in each 10 ml of buffer. The homogenate was centrifuged at 3,000 g for 10 min (4°C). An aliquot of the supernatant (total protein homogente) was quickly frozen and stored at −70°C. The remaining supernatant was centrifuged at 100,000 g for 90 min (4°C). The membrane pellet was suspended in 100 µl of buffer solution containing 10 mM Tris-HCl, 10 mM EDTA, 0.5 mM PMSF, 10% glycerol (vol/vol), and 50 mM β-mercaptoethanol (pH = 7.4) and stored at −70°C until use. Samples were separated in SDS-polyacrylamide gels according to the method of Laemmli (36). The blotting procedures were carried out according to Towbin et al. (69). Separate membranes were incubated with mouse monoclonal anti-SMA antibody (Sigma), mouse monoclonal anti-α1 (G1; kindly provided by Dr. M. Caplan, Yale School of Medicine, New Haven, CT) or mouse monoclonal anti-α2 antibody (McB2; kindly provided by Dr. K. J. Sweadner, Massachusetts General Hospital, Boston, MA). Blots were developed by enhanced chemiluminescence (PerkinElmer Life Sciences) with horseradish peroxidase-conjugated secondary antibodies (anti-mouse IgG H&L chain, goat; KPL). Films (Kodak BioMax ML) were placed in contact with the membranes in cassettes containing intensifying screens, and four to five plates with different exposure times were used to avoid film saturation as indicated by Miecha et al. (42). Computer scanning densitometry analysis quantified the signal intensity present in each lane.

Statistical analysis. Values are expressed as means ± SE; n denotes the number of animals studied. Male and female groups were analyzed by sex (male vs. female) and experimental treatment with a two-way ANOVA with sex (male vs. female) followed by Student-Newman-Keuls test to distinguish significant differences among the means of male and female data groups. Statistical evaluation of the contractile data was carried out by one-way ANOVA. The EC50 of SNP or...
ACh was determined from log-probit plots of the individual response vs. concentrations, and data are shown as averages of the individual values. Difference was considered statistically significant for $P < 0.05$.

RESULTS

Effect of ACh on Na$^+$-K$^+$-ATPase functional activity in aortic rings of male and female rats. It is well established that female rats have a greater sensitivity to the vasodilatory effect of ACh compared with male rats (56, 71). To assess the contributions of the Na$^+$ pump in the difference indicated above, we measured the effect of ACh on the function of Na$^+$-K$^+$-ATPase as ouabain-sensitive $^{86}$Rb/K uptake in rat aortic rings from both sexes. As shown in Fig. 1A, ACh (10$^{-6}$ M) significantly increased Na$^+$-K$^+$-ATPase function only in the aorta from female rats with intact endothelium (control females 206 ± 11 vs. 367 ± 29 mmol $^{86}$Rb/K/min$^{-1}$g wt tissue$^{-1}$ with ACh; $P < 0.01$). Removal of the endothelium abolished the response to ACh stimulation that was present in the female endothelium-intact aortic rings (Fig. 1B). No significant changes were observed in aortic rings from male rats in the presence of ACh, even though a tendency toward a diminished Na$^+$ pump activity was observed.

Effect of ACh on Na$^+$-K$^+$-ATPase of ovariectomized rats. To test whether estrogens are involved in the gender difference of ACh action on vascular Na$^+$-K$^+$-ATPase, we measured the enzyme functional activity in aortic rings from O VX rats. As shown in Fig. 2, ovariectomy (15 days) completely abolished the stimulation of the ouabain-sensitive $^{86}$Rb/K uptake elicited by ACh. When O VX rats received estradiol replacement therapy the effect of ACh on ouabain-sensitive $^{86}$Rb/K uptake was restored. Indeed, the increase in Na$^+$ pump was similar to the values observed in aortic rings from intact rats in the estrous period of the cycle. It should be noted that basal Na$^+$ pump functional activity is lower in O VX rats compared with normal female rats.

Effect of SNP in Na$^+$-K$^+$-ATPase functional activity of vascular tissue from male and female rats. The endothelium-dependent relaxation due to ACh is mainly related to NO synthesis. SNP is a NO donor that has been widely used in experimental studies. Therefore, we examined the effect of SNP on Na$^+$-K$^+$-ATPase in arteries from male rats and female rats in the estrous period of the cycle. Experiments were carried out in denuded aorta in the presence of 10 nM SNP. As shown in Fig. 3, gender differences in Na$^+$ pump functional activity were observed; SNP induced a significant increase in ouabain-sensitive $^{86}$Rb/K uptake only in female aortic tissue (control 123 ± 7 vs. 197 ± 12 mmol $^{86}$Rb/K/min$^{-1}$g wt tissue$^{-1}$ with SNP; $P < 0.05$).

Role of cGMP and PKG in SNP-induced increase in Na$^+$ pump functional activity. Methylene blue has been largely used to inhibit guanylate cyclase, the main effector mediating NO vasodilatory action (51). Recently KT-5823, a specific inhibitor of protein kinase G, has been described (31). Therefore, we measured the effect of both inhibitors in SNP action on Na$^+$ pump function. Experiments were carried out in denuded arteries from female rats incubated with SNP alone and in the presence of methylene blue or KT-5823. As shown in Fig. 4, both inhibitors completely blocked SNP-mediated increase in ouabain-sensitive $^{86}$Rb/K uptake.

![Fig. 1. Effect of ACh on ouabain-sensitive $^{86}$Rb/K uptake of male and female aortic rings. Endothelium-intact (A) or endothelium-denuded (B) aortic rings for male and female rats were preincubated with or with 1 mM ouabain. Ouabain-sensitive $^{86}$Rb/K uptake was calculated by subtracting uptake obtained in the presence of ouabain from uptake obtained in the absence of ouabain. Rings were incubated with 10$^{-6}$ M ACh or vehicle (control). Results are means ± SE of 9–12 experiments with each point assayed in triplicate. *P < 0.01 vs. basal condition.](image1)

![Fig. 2. Effect of ovariectomy and hormone replacement on ACh-mediated increase of the Na$^+$-K$^+$-ATPase activity of female aorta. Ovariectomized rats were kept with (OVX + E$_2$) or without (OVX) hormone replacement therapy as indicated in METHODS. Ouabain-sensitive $^{86}$Rb/K uptake was measured under basal conditions (open bars) or in the presence of 10$^{-6}$ M ACh (filled bars). Values are expressed as % of a paired aortic ring from a female estrous rat assayed in parallel (control). Results are means ± SE of 7 experiments with each point assayed in triplicate. E$_2$, 17β-estradiol. *P < 0.05, **P < 0.01 compared with control value of estrous female.](image2)
Fig. 3. Effect of sodium nitroprusside (SNP) on ouabain-sensitive $^{86}$Rb/K uptake in denudated male and female aorta. Aortic rings without endothelium were preincubated (10 min) with 10 nM SNP or vehicle (control). $^{86}$Rb/K uptake was determined in the presence or absence of $10^{-5}$ M ouabain to obtain the ouabain-sensitive component. Data are means ± SE of 6 experiments done in triplicate. *P < 0.05, basal vs. SNP rings.

**Role of Na$^+$/K$^+$-ATPase in vasodilatory response to SNP and ACh.** To evaluate the contribution of the Na$^+$ pump to SNP-induced relaxation, aortic rings were pretreated with ouabain ($10^{-3}$ M) for 20 min or solvent alone (control). Figure 5 summarizes cumulative concentration-response curves for SNP-induced relaxation ($10^{-10}$–$10^{-5}$ M SNP) in endothelium-denuded aortic rings from male and estrous female rats. No significant gender differences in the relaxation properties of SNP were observed when aortic rings were incubated in ouabain-free medium (EC$_{50}$: females 27.1 ± 12.2 nM vs. males 30.3 ± 9.9 nM). In the presence of ouabain, the vasodilatory response to SNP was significantly lower in aortic rings isolated from estrous female rats over the entire concentration range of SNP tested (Fig. 5B; P < 0.05). In female aortic rings sensitivity to SNP decreased in presence of ouabain (EC$_{50}$: 63.6 ± 12.7 nM vs. control 27.1 ± 12.2 nM; P < 0.05). In contrast, a minor ouabain effect was detected in male rat aorta, in which relaxant responses to SNP decreased significantly only at $10^{-7}$ M SNP (Fig. 5A; P < 0.05). In male, sensitivity (EC$_{50}$) to SNP did not differ significantly in presence of ouabain (57.2 ± 18.1 nM vs. control 30.3 ± 9.9 nM). There were no significant differences in the initial PE tension between male and female rats (812 ± 189 and 806 ± 62 mg/mg ring wt, respectively) or in presence of ouabain between male and female rats (675 ± 206 and 895 ± 53 mg/mg ring wt, respectively).

We also tested the effect of ouabain on ACh-mediated vasorelaxation. As shown in Fig. 6, relaxation was significantly impaired by ouabain in male and female rat aortic rings (P < 0.05). However, the ouabain effect was proportionally greater in tissues isolated from female compared with male rats (P < 0.01). In female aortic rings the sensitivity (EC$_{50}$) to ACh decreased in presence of ouabain (121.1 ± 19.9 nM vs. control 61.4 ± 8.1 nM; P < 0.05), whereas sensitivity (EC$_{50}$) in male aortic rings was unchanged in presence of ouabain (65.8 ± 44.9 nM vs. control 83.4 ± 21.1 nM).

**Gender differences on Na$^+$/K$^+$-ATPase catalytic isoform protein abundance in arterial tissue.** Gender differences in the Na$^+$ pump functional response to ACh could be related to a differential expression of the catalytic α-subunit isoforms of the Na$^+$--K$^+$--ATPase in rat aorta (11). Therefore, we examined total α$_1$ and α$_2$ protein present in aorta of male and estrous female animals. Crude membrane fractions obtained from endothelium-denuded arteries of male and female rats were analyzed by Western blot. The results are included in Fig. 7, expressed as relative abundance. The results were corrected by SMA (smooth muscle-specific marker) content present in the total protein homogenate before the isolation of the membrane fraction to eliminate possible differences in the cellular composition of female vs. male aortic tissue. Significant gender differences in α catalytic protein abundance for both isoforms were observed. Female rats have lower levels of α$_1$ catalytic isoform than male rat aorta (P < 0.05). The opposite was true for the α$_2$ catalytic isoform; female aorta had 44% more α$_2$ than male tissue (P < 0.05). No significant differences in SMA content of male vs. female aorta were detected.

**Estradiol-induced expression of α$_2$ isoform of Na$^+$/K$^+$-ATPase.** Direct evidence of α$_2$ mRNA abundance upregulation by estradiol in arterial tissue was obtained by the effect of this hormone on male arterial explants. Aorta and also mesenteric arteries were studied because they are considered muscular arteries, with resistance branches affecting total peripheral resistance and blood pressure in vivo. As shown in Fig. 8D, 24-h incubation with 1 nM estradiol induced an increase in α$_2$ mRNA abundance of aortic explants (2 times control; P < 0.05). Figure 8B includes α$_2$ mRNA levels after 24-h incubation with estradiol in male mesenteric explants. Similar results were observed when testing α$_2$ mRNA in mesenteric arteries, in which an increase of 1.9 times was observed compared with controls at 0.1 nM estradiol (P < 0.05).

**DISCUSSION**

There is ample evidence that Na$^+$/K$^+$-ATPase, which contributes to the maintenance of an electrochemical gradient of Na$^+$ and K$^+$ across the cell membrane, plays an important role in the regulation of vascular smooth muscle tone (1, 8, 10, 14, 24, 66). An increase in Na$^+$ pump activity may induce smooth muscle relaxation by increasing Na$^+$/Ca$^{2+}$ exchange and re-

![Fig. 4. Effect of inhibitors of the NO/cGMP pathways on the SNP-induced stimulation of Na$^+$/K$^+$-ATPase activity.](http://ajpheart.physiology.org/)

*Fig. 4. Effect of inhibitors of the NO/cGMP pathways on the SNP-induced stimulation of Na$^+$/K$^+$-ATPase activity. Data represent ouabain-sensitive Na$^+$/K$^+$-ATPase activity in female aorta after incubation with vehicle (control), $10^{-8}$ M SNP alone, SNP + $1 \mu$M methylene blue (MB), or SNP + 1 $\mu$M KT-5823. Methylene blue was added 20 min before SNP was added, and, when pertinent, KT-5823 was added 15 min in advance. Data are means ± SE for triplicate samples of 6 experiments. *Significantly different from all other groups (P < 0.05).*


producing Ca$^{2+}$ influx through membrane potential-dependent Ca$^{2+}$ channels (1, 9, 10, 14). In the present study we found that the incubation of arterial smooth muscle with ACh significantly increased ouabain-sensitive $^{86}$Rb/K uptake in the female rat aorta, whereas no effect was observed in male aortic rings. The increase in Na$^+-$K$^+$-ATPase activity in response to ACh was only observed in intact arteries, suggesting a direct influence of an endothelial factor. As we showed previously (41), the relative contribution of the endothelium itself in the uptake of ouabain-sensitive $^{86}$Rb/K from intact aortic rings is negligible, therefore suggesting that an endothelial factor is directly involved in Na$^+$ pump activation.

The aorta, as well as other arterial beds of female rats, appears to produce a greater amount of NO compared with those of males (3, 13, 25, 62). The importance of the enhanced NO production in females has not been fully elucidated. White et al. (71) showed that female arteries have an augmented ACh-stimulated NO production compared with that of males. In addition, ACh stimulates the production of a non-NO endothelium-derived hyperpolarizing factor to a greater extent in females than in males (21, 56). Previous studies carried out in cultured vascular smooth muscle cells showed an endothelial stimulating factor of the Na$^+$ pump (52). To obtain further insight in the eventual participation of NO on the different gender response to ACh, we have performed a series of experiments in the presence of an exogenous NO donor. We found male-female differences in SNP-mediated Na$^+$ pump activity similar to those observed in the presence of ACh; these results indicate gender differences in the mechanism of action of NO itself.

Vascular relaxation in response to nitrovasodilators involves signal transduction from NO exposure to cGMP synthesis via activation of soluble guanylate cyclase. Once stimulated, guanylate cyclase generates cGMP, which in turn causes smooth muscle relaxation (5, 49, 57). cGMP has been shown to either activate or inhibit Na$^+$-K$^+$-ATPase activity (16, 20, 22, 65). In the present study, SNP enhancement of the Na$^+$ pump in female rat aorta was abolished by KT-5823, a specific cGMP-dependent protein kinase inhibitor (31). Also, methylene blue (51) suppressed the SNP-stimulated Na$^+$ pump. In contrast to the findings of the present study, Gupta et al. (22) showed that NO stimulates Na$^+$-K$^+$-ATPase activity independently of its ability to increase intracellular cGMP in human corpus cavernosum. In addition, carbachol-induced inhibition of Na$^+$-K$^+$-ATPase activity in choroid plexus via the NO/cGMP pathway was shown by Ellis et al. (20). However, Tamaoki et al. (65) found that accumulation of intracellular cGMP correlates with activation of Na$^+$-K$^+$-ATPase in pulmonary artery smooth muscle cells. Cogolludo et al. (16) showed that SNP reduces intracellular calcium and causes relaxation through activation of Na$^+$-K$^+$-ATPase in mesenteric piglets arteries. Rapoport et al. (50) suggested the possibility that SNP could have a stimulatory effect in male rat aorta. Pontiggia et al. (48) showed that cGMP inhibits the Na$^+$-K$^+$-ATPase of brain endothelial cells by acting on the $\alpha_3$-isoform, whereas $\alpha_2$ and $\alpha_3$ are hardly affected. These data suggest that inhibition of Na$^+$-K$^+$-ATPase by cGMP occurs in an isoform-selective manner.

Na$^+-$K$^+$-ATPase is a heteromeric enzyme composed of two polypeptide subunits: a 112-kDa $\alpha$-subunit and a 40- to 60-kDa glycosylated $\beta$-subunit. The $\alpha$-subunit contains the cation and ATP binding sites to perform the catalytic and transport activity of the enzyme. Four $\alpha$-subunits ($\alpha_1$, $\alpha_2$, $\alpha_3$, and $\alpha_4$) and three $\beta$-subunits ($\beta_1$, $\beta_2$, and $\beta_3$) have been found. In some tissues the enzyme is associated with another protein, named $\gamma$ (67). We previously established (42) the Na$^+$-K$^+$-ATPase isoforms present in rat vascular tissue. In rat aorta, we found that only the $\alpha_2$ and $\alpha_3$ catalytic isoforms are present (42). The $\gamma$ protein is absent in this tissue (unpublished observations). We have now determined that there are significant gender differences in the mechanism of action of NO in the female rat aorta. Arteries were preconstricted with $10^{-6}$ M phenylephrine. Ouabain significantly decreased the sensitivity to SNP in the concentrations indicated by asterisks: $*P < 0.05$, $**P < 0.001$. Each data point represents mean ± SE of 6 experiments; points without error bars have SE smaller than symbol size.

\[ \frac{\text{% Initial tension}}{\text{Sodium nitroprusside (log M)}} \]

\[ \frac{\text{% Initial tension}}{\text{Sodium nitroprusside (log M)}} \]

Fig. 5. SNP concentration-response curves in endothelium-denuded aorta from male (A) and female (B) rats in the presence or absence (control) of 1 mM ouabain. Arteries were preconstricted with $10^{-6}$ M phenylephrine. Ouabain significantly decreased the sensitivity to SNP in the concentrations indicated by asterisks: $*P < 0.05$, $**P < 0.001$. Each data point represents mean ± SE of 6 experiments; points without error bars have SE smaller than symbol size.

\[ \frac{\text{% Initial tension}}{\text{Acetylcholine (log M)}} \]

\[ \frac{\text{% Initial tension}}{\text{Acetylcholine (log M)}} \]

Fig. 6. ACh-response curves in endothelium-intact aorta from male (A) and female (B) rats in the presence or absence (control) of 1 nM ouabain. Arteries were preconstricted with $10^{-6}$ M phenylephrine. Ouabain significantly decreased the sensitivity to ACh in the concentrations indicated by asterisks: $*P < 0.05$, $**P < 0.001$. Each data point represents mean ± SE of 3 experiments; points without error bars have SE smaller than symbol size.
An interesting finding of the present study was the observation that, in vitro, estradiol upregulates α2 mRNA in male vascular tissue. It is known that the estrogen receptor (ER) interacts with DNA by direct binding to sequences related to the palindromic 5′-aGGTCAnmTGACCt-3′, by tethering to other proteins bound at simian virus 40 protein-1 (Sp1) and activator protein-1 (AP-1) sites and through interaction with genes containing isolated GGTCA half-sites or direct repeats (18, 19, 34, 46, 47). Analysis of the promoter region (~4000 bases relative to the start translation site, base 156,863,422 of chromosome 1; accession number J05096, GenBank) of the human α2 gene with Transcription Element Search Software (57a) demonstrated the absence of classic palindromic estrogen-responsive elements. However, several G-C-rich boxes were identified (GGCGGG at positions −653, −857, −1243, −2363). Putative Sp1 binding sites were abundant in the entire promoter region and in proximity (59–80 bases) to the regions enumerated above. The induction by estrogen of cathepsin D gene expression in MCF-7 human breast cancer cells by ER-Sp1 complexes (35) occurs through the interaction of similar DNA domains, suggesting that these regions could represent potential ER-dependent regulatory regions. Also, numerous potential AP-1 (Fos/Jun) binding sites were identified in the region, in accordance with the original characterization of the human gene, Na,K-ATPase α2 (59), which could also represent estrogen-dependent regulatory sites.

Participation of Na+−K+−ATPase in NO-induced relaxation was evaluated indirectly by measuring SNP relaxation proper-

Fig. 7. Western blot analysis of α1- and α2-protein isofrom levels in male and female rat aorta. Denuded thoracic aorta from 4 animals/group in each set of experiments (6 experiments) Crude membrane fractions (10% of total protein homogenate was subjected to SDS-PAGE and blotted as described in METHODS. A: representative immunoblot with anti-α1 monoclonal antibody (top) and smooth muscle actin antibody (SMA; bottom). B: means ± SE of α1 protein levels normalized to SMA protein levels. *P < 0.05. C: representative immunoblot of aortic membrane with anti-α2 monoclonal antibody and SMA (total protein homogenate) D: means ± SE of α2 levels normalized to SMA protein levels in each separate experiment; *P < 0.05 (n = 6).

differences in the relative amount of α1 and α2 catalytic isoforms of Na+−K+−ATPase. Female arterial vessels have greater α2 protein abundance than males, without changes in basal ouabain-sensitive 86Rb/K uptake. As we showed previously (2, 41) basal Na+−K+−ATPase activity is related not only to the total amount of protein present but to the number of catalytic units in the plasma membrane and/or intracellular posttranslation modifications. In the aorta of streptozotocin-induced diabetic rats, we demonstrated (41) a diminished expression of α1 protein without change in α2-mediated 86Rb/K uptake, whereas the reduction of α2 protein accounted for the reduction of total Na+ pump activity of diabetic animals. α2-Isomorf activity is regulated by several hormones (7, 38, 42, 44, 66). Furthermore, the regulation of α2-isoform activity has been proposed as part of the mechanism of action of insulin and other vasoactive substances in vascular smooth muscle cells (15, 41). A greater ratio of α2 to α1 in female compared with male aorta could probably explain the fact that ACh or NO donor is capable of significantly increasing Na+−K+−ATPase activity in female arterial tissue. A reticular distribution of the α2-isoform has been demonstrated in the plasma membrane of arterial myocytes in culture, paralleling the endoplasmic reticulum (10, 29). This led to the proposal that the α2-isoform could regulate intracellular Na+ concentration in a restricted cytosolic space, affecting membrane potential and local Ca2+ concentration at this cellular region (29). Recently James et al. (27) showed that hearts from mice with genetically reduced levels of α2-isoform are hypercontractile as a result of increased Ca2+ transients during the contractile cycle.

Fig. 8. α2-Isomorf mRNA expression levels in rat arterial tissue. Arterial explants were placed in culture for 24 h with the concentration of estradiol indicated or vehicle alone (control). Semiquantitative RT-PCR analysis was performed with 0.5 μg of total SMA segments of arterial tissue. Isoform-specific α2 mRNA was assayed with primers designed to yield a 236-bp product. PCR amplification of the 18S ribosomal RNA served as an internal control to yield a 312-by product. Details are described in METHODS. A and C: representative analysis of mesenteric artery (A) and aorta (C). Bar indicates the relative abundance normalized by 18S ribosomal RNA, which served as an control. B: mesenteric artery (n = 3 or 4). D: aorta (n = 4). *P < 0.05 vs. control.
ties in the presence of 1 mM ouabain to completely inhibit Na\textsuperscript{+}-K\textsuperscript{+}-ATPase. Na\textsuperscript{+} pump inhibition by ouabain markedly decreased the response to SNP in female rat aorta compared with male rat aorta. As shown by the results, no significant gender differences in the relaxation properties of SNP were observed when aortic rings were incubated in ouabain-free medium, a result that agrees with data previously reported by others (30). As previously reported, we confirmed gender differences in ACh-induced relaxation (Fig. 6). In addition, we demonstrated that ouabain markedly reduces the ACh vasodilatory effect and the digitalis inhibitory effect is significantly greater in female than male rats. Therefore, the difference observed in the presence of ouabain suggests a gender-dependent vasodilatory mechanism secondary to estrogen action.

The findings of this study may have several clinical implications. For example, microvascular Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity was reported to be reduced in animal models of diabetes in a sex-dependent manner, with activity being reduced in males but not females (60), suggesting that females are protected from the decline in vascular ATPase activity, likely because of estrogen-induced activation and expression of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase. In summary, we found that ACh induces activation of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase in female rat arteries, an effect mediated by the presence of estrogens. The increased ACh-mediated effect in females could be related to differences in the amount of \(\alpha_1\) and \(\alpha_2\) catalytic subunits of the Na\textsuperscript{+} pump present in female vs. male rats. Estradiol induces \(\alpha_2\) expression in male arterial tissue. Because Na\textsuperscript{+}-K\textsuperscript{+}-ATPase is involved in the plasma membrane potential of vascular smooth muscle cells, our studies in SNP-mediated relaxation in the presence of ouabain provide evidence that enhanced vasodilation in arteries from female rats may implicate increased vascular Na\textsuperscript{+} pump activity.

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