

## A profound decrease in maternal arginine uptake provokes endothelial nitration in the pregnant rat

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**Reshef R, Schwartz D, Ingbir M, Shtabsky A, Chernichovski T, Isserlin BA, Chernin G, Levo Y, Schwartz IF.** A profound decrease in maternal arginine uptake provokes endothelial nitration in the pregnant rat. *Am J Physiol Heart Circ Physiol* 294: H1156–H1163, 2008. First published December 21, 2007; doi:10.1152/ajpheart.01051.2007.—While a specific role for nitric oxide (NO) in inducing the hemodynamic alterations of pregnancy is somewhat controversial, it is widely accepted that excess NO is generated during pregnancy. L-Arginine is the sole precursor for NO biosynthesis. Among several transporters that mediate L-arginine uptake, cationic amino acid transporter-1 (CAT-1) acts as the specific arginine transporter for endothelial NO synthase. The present study was designed to test the hypothesis that, during pregnancy, when arginine consumption by the fetus is significantly increased, compensatory changes in maternal arginine uptake affect the endothelium. Uptake of radiolabeled arginine (L-[<sup>3</sup>H]arginine) by freshly harvested maternal aortic rings from pregnant rats decreased by 65 and 30% in mid- and late pregnancy, respectively, compared with those obtained from virgin animals. This decrease was associated with a significant increase in endothelial protein nitration (the footprint of peroxynitrite generation), as shown by both Western blotting and immunohistochemistry utilizing anti-nitrotyrosine antibodies, reflecting endothelial damage. Northern blot analysis revealed that steady-state aortic CAT-1 mRNA levels did not change throughout pregnancy, whereas CAT-1 protein abundance was significantly increased, peaking at mid-pregnancy. Protein content of protein kinase C (PKC)- $\alpha$ , which was previously shown to decrease CAT-1 activity, increased significantly in the pregnant animals and was associated with a significant increase in CAT-1 phosphorylation. Intraperitoneal injection of  $\alpha$ -tocopherol, a PKC- $\alpha$  inhibitor, prevented the decrease in arginine transport and attenuated protein nitration. In conclusion, aortic arginine uptake is reduced during pregnancy, through posttranslational modulation of CAT-1 protein, presumably via upregulation of PKC- $\alpha$ . The aforementioned findings are associated with an increase in protein nitration and, therefore, in selected individuals, may lead to the development of certain forms of endothelial dysfunction, like preeclampsia.

nitric oxide; endothelial function; arginine; pregnancy

NUMEROUS STUDIES HAVE EMPHASIZED a pivotal role for nitric oxide (NO) in mediating endothelial-induced vasorelaxation in pregnancy (10, 33). Since the capacity of the constitutive, Ca<sup>2+</sup>/calmodulin-sensitive NO synthase (eNOS) to generate adequate quantities of NO is the hallmark of endothelial function, it was more than natural that alterations in NO generation have been used to explain the mechanisms involved in the systemic and renal vasodilatation, characteristic of nor-

mal pregnancy, as well as the dysfunction of the endothelium observed in preeclampsia (4, 21, 34). Whereas a specific role for NO in inducing these events is somehow controversial, there is evidence supporting the notion that increased NO is produced during pregnancy (4–6). Augmented levels of cGMP, a major second messenger of NO, as well as the stable metabolites of NO (NO<sub>2</sub>/NO<sub>3</sub>) were demonstrated during pregnancy in rats (5, 6). Moreover, chronic inhibition of NO synthesis prevented pregnancy-induced systemic and renal hemodynamic changes (4). Recently, it has been reported that eNOS activation during pregnancy in uterine artery endothelial cells is regulated by Ca<sup>2+</sup> concentration and multiple kinases (30). Unfortunately, the status of NO synthesis in pregnant women is less clear (7).

Being the sole precursor for NO synthesis, altered arginine metabolism has been used to explain various disorders associated with NO biosynthesis. Among several transport systems that mediate L-arginine uptake (y<sup>+</sup>, b<sup>0,+</sup>, B<sup>0,+</sup>, and y<sup>+L</sup>), system y<sup>+</sup> is widely expressed and considered to be a major arginine transporter in most tissues and cells (14, 32). Encoded by cationic amino acid transporters (CAT)-1, CAT-2, and CAT-3, system y<sup>+</sup> is characterized by high affinity for cationic amino acids, sodium independence, and stimulation of transport by substrate on the opposite (trans) side of the membrane (31).

Accumulated evidence from our laboratory and others suggests that each transporter has affinity to a specific NO synthase (NOS) isoform (18, 22–25, 27). More specifically, CAT-1 is considered as the predominant arginine supplier to eNOS. CAT-1 and eNOS were found to colocalize in a caveolar complex (18), and suppression of endothelium-mediated microvascular dilatation by dexamethasone involves downregulation of both eNOS and CAT-1 (22). Moreover, these two proteins are regulated by protein kinase C (PKC)- $\alpha$  (18, 19). We have suggested that increased arginine uptake through upregulation of CAT-1 may contribute to the pathogenesis of diabetic hyperfiltration, via activation of eNOS (25). It was recently reported that, in a rat model of chronic renal failure, arginine uptake is attenuated due to a decrease in CAT-1 protein content (24). Several studies have investigated arginine metabolism at gestation. Speake et al. (28) found an increase in arginine uptake by the basal plasma membrane of the syncytiotrophoblast in vitro and suggested that a similar increase in arginine transport in vivo could alter the delivery of arginine to the syncytiotrophoblast endothelium. In contrast, peripheral

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blood mononuclear cells from pregnant women exhibited an augmented arginine uptake through upregulation of CAT-2 (17). Careful examination of the aforementioned experimental data reveals that they do not address the issue of arginine availability in the maternal vasculature. To do so, maternal endothelium rather than a whole placenta or peripheral leukocytes should be the tissue of reference. By looking at aortic arginine uptake and protein nitration as a footprint of peroxynitrite-protein interaction, the experiments reported herein were designed to test the hypothesis that alterations in arginine uptake during pregnancy impact the maternal endothelium and to elucidate a molecular mechanism to explain these observations.

## METHODS

**Materials.** All standard reagents were obtained from Sigma (St. Louis, MO), unless indicated otherwise. L-[<sup>3</sup>H]arginine was supplied by Perkin Elmer Life and Analytical Sciences (Boston, MA).

**Animals and surgical preparation.** The experiments in this study were approved by both the institutional (Tel Aviv Sourasky Medical Center) and national ethics committees for animal research guide for care, and all animal experiments described in this study were conducted according to the guide of care and use of animals protocol and approved by the institutional committee on ethics in animal experiments. Studies were performed using female Wistar rats at 12–14 wk of age. Rats destined to become pregnant were housed with a male breeder in a climate-controlled room with a 12:12-h light-dark schedule. *Day 1* of pregnancy was documented by the presence of spermatozoa in the vaginal lavage. Pregnant rats at 12–14 and 20–22 days of gestation and age-matched virgin controls were used for the experimental procedures. Additional animals were given intraperitoneal injections of  $\alpha$ -tocopherol, 90 mg/kg body wt, every other day, or castor oil as a vehicle, starting from *day 1* of pregnancy or for a corresponding length of time in virgin animals. Rats were euthanized using CO<sub>2</sub>.

**L-Arginine uptake by aortic rings.** Uptake of radiolabeled L-arginine in the rat aorta was measured according to previously described methods, with modification (24). Immediately after death, the aorta was carefully excised from the left renal artery to the aortic valve ring and placed in ice-cold HEPES buffer. The vessels were dissected free from adherent connective tissue and cut into rings (length 3–4 mm).

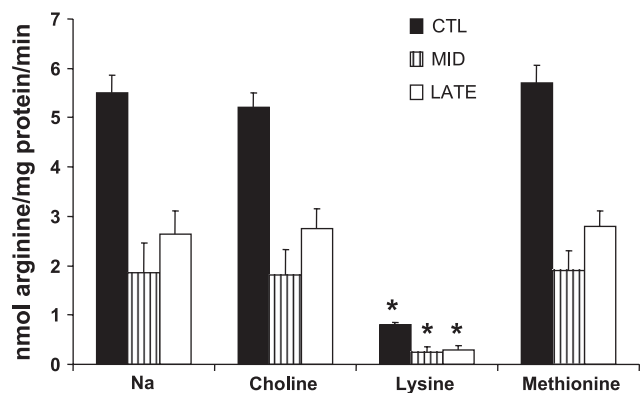


Fig. 1. Sodium independence and *cis* inhibition (competitive) by lysine of aortic arginine uptake in pregnant rats. Uptake of radiolabeled arginine (L-[<sup>3</sup>H]arginine) by freshly harvested aortic rings from virgin and pregnant animals is shown in the presence of either 140 mM sodium chloride or 140 mM choline chloride with either lysine or methionine (10 mM) added to the transport solution. Values are means  $\pm$  SE of 4 different experiments. \**P* < 0.05 vs. sodium chloride. Ctl, virgin rats; Mid, *days 12–14* of pregnancy; Late, *days 20–22* of pregnancy.

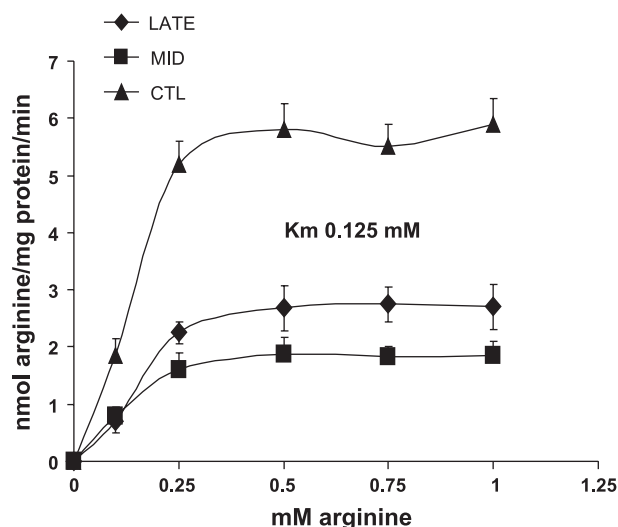


Fig. 2. Concentration dependence of L-arginine uptake by aortic rings of pregnant rats. Uptake of [<sup>3</sup>H]arginine was measured for 1 min in freshly harvested aortic rings from virgin and pregnant rats over a range of concentrations (0–1 mM). Values are means  $\pm$  SE of 4 different experiments.

Each segment was cut longitudinally in half. To determine arginine transport, aortic segments of each experimental group were incubated and shaken for 10 min in HEPES buffer at pH 7.4, 37°C. L-[<sup>3</sup>H]arginine and L-arginine, in a final concentration of 1 mM, were added to a total volume of 2 ml for an additional 1 min. The duration of 1 min was chosen since it was within the linear portion of uptake curves (data not shown). Transport was terminated by rapidly washing the aortic rings with ice-cold PBS buffer (4 times, 3 ml/tube). The rings 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 by liquid scintillation spectrometry (Betamatic, Kontron). The remaining 300  $\mu$ l were used for protein content determination by the Lowry method (Lowry assay kit, Sigma). To correct for nonspecific uptake or cell membrane binding, additional studies were performed in which aortic segments were incubated with 10 mM unlabeled arginine in HEPES buffer, and the associated radioactivity was subtracted from each data point. At least five animals were used for each experimental group.

**Northern blot analysis.** CAT-1 mRNA level was determined by Northern hybridization. Twenty micrograms of total RNA were denatured and fractionated by size on 1.3% formaldehyde-agarose gel.

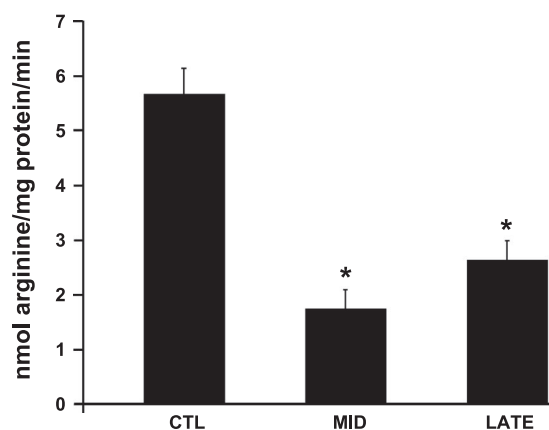
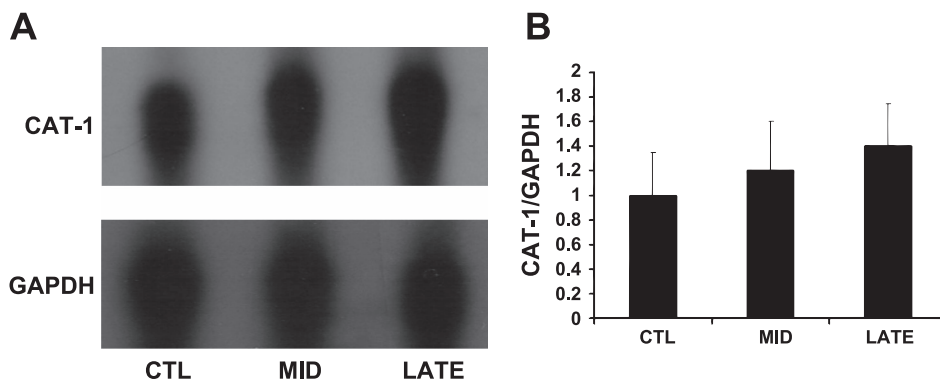


Fig. 3. Uptake of radiolabeled arginine (L-[<sup>3</sup>H]arginine) (1 mM) by aortic rings freshly harvested from the various experimental groups. Values are means  $\pm$  SE of at least 5 different experiments. \**P* < 0.01 vs. Ctl.

Fig. 4. *A*: Northern blot analysis of aortic cationic amino acid transporter (CAT)-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA from all experimental groups. These blots are representative of four different experiments. *B*: graphic representation of relative changes in mRNA levels. The ratio of CAT-1 mRNA (7.9 kb) over GAPDH mRNA in aortic rings was plotted against the appropriate experimental groups. Values are means  $\pm$  SE of 4 different experiments.



RNA was transferred overnight, by capillary action, to a nylon membrane (Hybond-N, Amersham International, Buckinghamshire, UK), and cross-linked by short-wave ultraviolet illumination. Purified end products of CAT-1 and GAPDH cDNA (High Pure PCR Product Purification Kit, Boehringer, Mannheim, Germany) were used directly for radiolabeling with [ $^{32}$ P]dCTP by a random primer labeling method (Random Primers DNA Labeling System Kit, Gibco BRL, Gaithersburg, MD). After 1 h at 50°C, membranes were hybridized overnight at 50°C with the  $^{32}$ P-labeled cDNA. Membranes were sequentially washed twice in 1 $\times$  standard sodium citrate (SSC), 0.1% SDS for 15 min, at room temperature, once in 1 $\times$  SSC, 0.1% SDS for 15 min at 50°C, followed by 0.5 $\times$  SSC, 0.1% SDS at 55°C for 15 min, and then washed at high stringency in 0.1 $\times$  SSC, 0.1% SDS at 57°C for 15 min. Autoradiography was carried out with Kodak XAR film (Kodak, Rochester, NY) for 24–48 h at  $-80^{\circ}\text{C}$ . Relative mRNA abundance was quantified by measuring the density of the exposed film with a densitometer (B.I.S 202D) (Fuji, Tokyo, Japan).

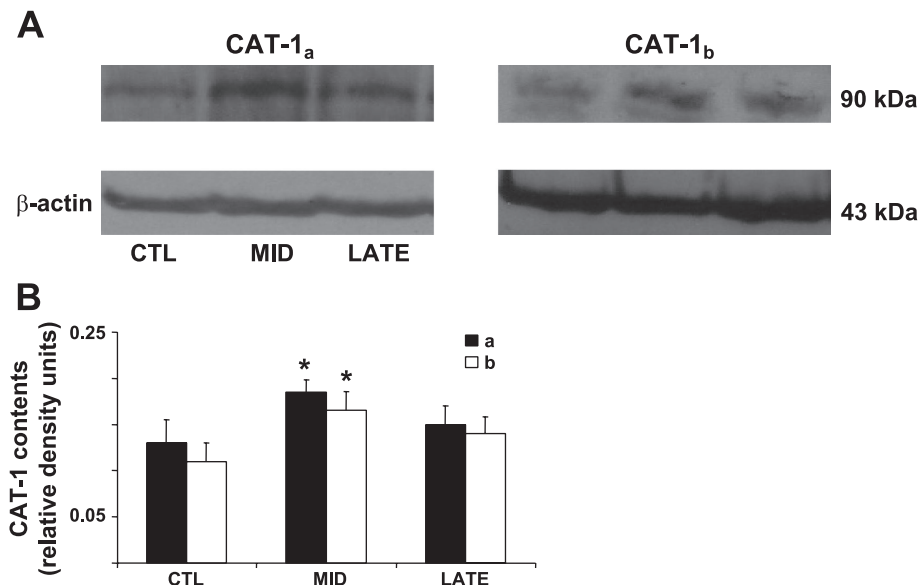
CAT-1 mRNA level was normalized to GAPDH mRNA and expressed in arbitrary units as the ratio of CAT-1 to GAPDH expression, in four different experiments.

**Protein quantification by Western blotting.** Aortic CAT-1 and PKC- $\alpha$  protein expression were determined by immunoblotting. Briefly, excised aortas were separately placed in ice-cold PBS lysis buffer (pH 7.4), containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 4.5  $\mu\text{M}$  leupeptin, and 5  $\mu\text{M}$  aprotinin) (ICN Biomedicals), 0.01% Triton X-100, and 0.1% SDS, and 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  $-70^{\circ}\text{C}$ . 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  $\mu\text{g}$ ) 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-Tween 20 (PBS-T) containing 5% nonfat dried milk, at room temperature. Membranes were then incubated with polyclonal rabbit anti-rat CAT-1 antibodies, 1:500 and 1:200 for peptide-1 and peptide-2, respectively (synthesized by Dr. O. Leitner, Weizmann Institute, Rehovot, Israel), mouse anti-rat PKC- $\alpha$  antibodies, or monoclonal mouse anti-rat nitrotyrosine antibodies (both 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- $\beta$ -actin antibodies as an internal control. The reactive bands corresponding to CAT-1, PKC- $\alpha$ , and nitrotyrosine were detected by enhanced chemiluminescence (Kodak X-OMAT AR film) and quantified by densitometry ( $n = 3$  different experiments).

**Immunoprecipitation studies.** Aliquots of aortic tissue cell lysate (1 ml) from the different experimental groups were used for immunoprecipitation. Each tissue lysate sample was incubated with 20  $\mu\text{l}$  of anti-CAT-1 antibodies for 2 h at 4°C. Optimal antibody concentration was determined by titration. This was followed by addition of 20  $\mu\text{l}$

Fig. 5. *A*: representative Western blot analysis showing regulation of CAT-1 protein level, using two different anti-CAT-1 antibodies (CAT-1<sub>a</sub> and CAT-1<sub>b</sub>) in freshly harvested aortic rings from the same experimental groups. *B*: densitometric analysis of aortic CAT-1 contents from the various experimental groups. Each bar represents the mean of the relative density units  $\pm$  SE from 4 different experiments. \* $P < 0.05$  vs. Ctl.



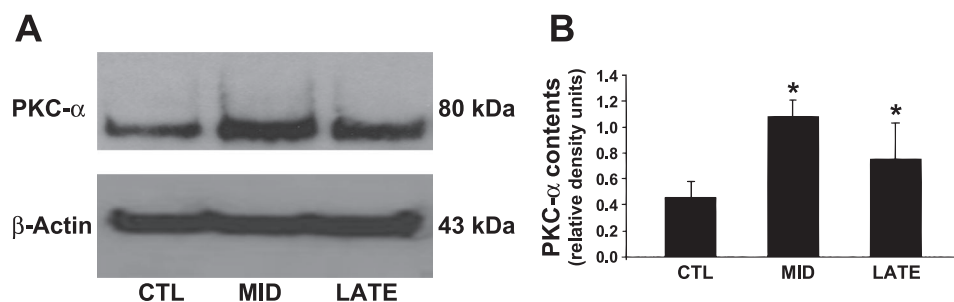


Fig. 6. *A*: representative Western blot analysis showing regulation of PKC- $\alpha$  protein level in freshly harvested aortic rings from the same experimental groups. *B*: densitometric analysis of PKC- $\alpha$  contents of the experiments shown in *A*. Each bar represents the mean of the relative density units  $\pm$  SE from 3 different rats. \* $P < 0.05$  vs. Ctl.

of protein A-agarose (Santa Cruz) and incubation overnight at 4°C on a rotating device. Pellets were collected by centrifugation at 3,000 rpm for 30 s, 4°C. The supernatants were discarded, and each pellet was subsequently washed three times with PBS. After final wash, the pellets were resuspended in 40  $\mu$ l of 2 $\times$  electrophoresis sample buffer, boiled for 3 min, and subjected to immunoblotting with antibodies against CAT-1 or the phosphorylated tyrosine residue of CAT-1 (Santa Cruz Biotechnology). Representative results of three separate experiments are shown. To estimate the phosphorylation of CAT-1 in the different groups, the density of bands for CAT-1 and its phosphorylated form on a film were analyzed as above. Results are adjusted for CAT-1 levels and expressed in arbitrary units (means  $\pm$  SE,  $n = 3$  rats).

**Immunohistochemical analysis.** Using additional animals, nitrotyrosine formation, a marker of peroxynitrite generation, was determined by immunohistochemical staining. Immunohistochemistry was performed by employing a mouse monoclonal antibody for the detection of nitrotyrosine (Clone 39B6, Santa Cruz, CA). Paraffin-embedded tissue sections (4  $\mu$ m) were mounted on SuperFrost Plus glass (Menzel-Glazer, Braunschweig, Germany) and processed by an automated immunostainer (VENTANA ES, Ventana Medical System, Tucson, AZ).

After deparaffinization, heat-induced antigen retrieval was performed at a controlled temperature in a microwave processor (H2800 model, Energy Beam Sciences) in 10 mM citrate buffer, pH 6.0, for 10 min at 97°C. Immunohistochemistry was performed using a three-step indirect process based on the labeled-(strept)avidin-biotin (LAB-SA) peroxidase complex method. Sections were incubated at a controlled temperature of 37°C for 32 min with 1:100 dilution of nitrotyrosine.

Automated immunostaining was performed using the I-View diaminobenzidine (DAB) detection kit (Ventana Medical System), according to a standard Ventana program. The I-View DAB detection kit utilizes biotinylated secondary antibodies to locate the bound primary antibody, followed by the binding of streptavidin-horseradish peroxidase conjugate. The complex is then visualized with hydrogen peroxidase substrate and 3,3'-DAB tetrahydrochloride chromogen, which produces a dark brown precipitate that is readily detected by light microscopy.

The sections were then counterstained with hematoxylin, dehydrated, and mounted for microscopic examination. Tissues from three rats of each experimental group were processed.

**Statistical analysis.** Data are presented as means  $\pm$  SE. One-way ANOVA was conducted for comparison between groups. Post hoc analysis using least significant difference algorithm was performed to allocate the source of significance.

## RESULTS

**Aortic arginine transport.** Initially, we wished to explore the possibility that pregnancy affects the characteristics of  $y^+$  system, the predominant arginine uptake system.

Figure 1 demonstrates that, in aortic rings harvested from pregnant rats, the arginine transport system remained sodium independent. Excess concentration of lysine strongly inhibited

L-arginine uptake, while the neutral amino acid methionine was found to be a poor inhibitor. In addition, we characterized the kinetics of L-arginine transport by measuring saturable uptake of L-arginine (0–1 mM). The plots of L-arginine uptake as a function of extracellular L-arginine concentration are shown in Fig. 2. A high-affinity transporter was found to be present with a  $K_m$  of 125  $\mu$ M. These data establish that, similar to virgin rats, in aortic rings from pregnant rats, system  $y^+$  remains the predominant arginine transport system, with kinetic properties similar to those of CAT-1 and CAT-2.

To explore a possible effect of gestation on  $y^+$  system, aortic arginine uptake was measured at mid- (days 12–14) and late (days 20–22) pregnancy. Compared with virgin animals, arginine transport was significantly decreased by 65 and 30% at these two time points, respectively ( $P < 0.01$ , Fig. 3).

**Regulation of aortic CAT-1 in pregnancy.** To determine whether these gestation-induced changes in arginine uptake are associated with parallel directional changes in mRNA levels for CAT-1, Northern blot analysis was performed. We found that steady-state aortic CAT-1 mRNA levels did not change throughout pregnancy (Fig. 4). To further explore a possible role for CAT-1 in arginine traffic in pregnancy, we examined CAT-1 protein levels. CAT-1 protein was identified as ~90 kDa. Utilizing two different anti-CAT-1 antibodies, we found

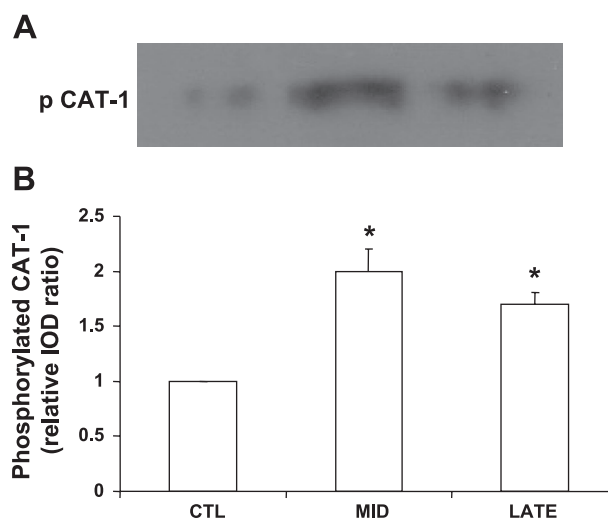


Fig. 7. *A*: representative immunoblot of aortic rings from 3 rats of each experimental group after immunoprecipitation of CAT-1. CAT-1 immunoprecipitate was blotted for phosphorylated (p) CAT-1. *B*: the relative integrated optical density (IOD) ratios of phosphorylated CAT-1 to CAT-1 in freshly harvested aortic rings from the various experimental groups. Each bar represents the mean  $\pm$  SE from 3 different rats. Data from control are normalized to 1. \* $P < 0.05$  vs. Ctl.

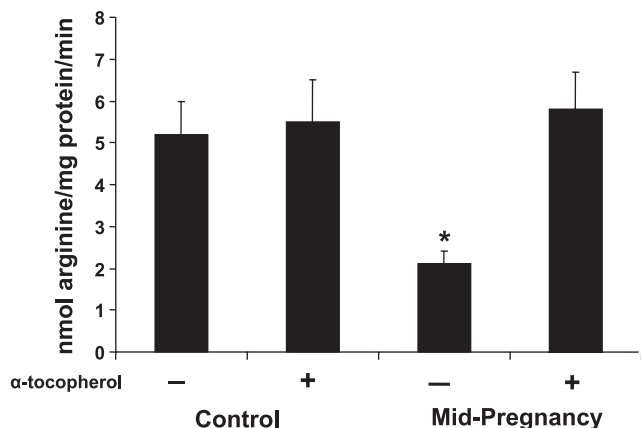


Fig. 8. Effect of  $\alpha$ -tocopherol, a PKC- $\alpha$  inhibitor (90 mg/kg body wt every other day) or castor oil as a vehicle, on arginine transport in freshly harvested aortic rings from virgin rats and animals at 12–14 days of pregnancy (mid-pregnancy). Values are mean  $\pm$  SE of at least 5 different experiments. \* $P < 0.05$  vs. untreated mid-pregnant rats.

that CAT-1 abundance was significantly increased in aortic rings harvested from pregnant rats. However, statistical significance was not found at late gestation (Fig. 5). To evaluate a possible posttranslational mechanism for CAT-1 inactivation in pregnancy, we performed Western blotting for PKC- $\alpha$ , which regulates CAT-1 activity. The abundance of PKC- $\alpha$  was significantly augmented in both mid- and late pregnancy (Fig. 6). Immunoprecipitation studies for phosphorylated CAT-1 in vir-

gin rats demonstrated a faint signal, which was significantly increased during mid- and late pregnancy (Fig. 7). To support the hypothesis that upregulation of PKC- $\alpha$  provokes the decline in arginine transport, rats at mid-pregnancy were treated with  $\alpha$ -tocopherol, a known inhibitor of PKC- $\alpha$  activity (90 mg/kg body wt every other day, starting from *day 1* of gestation) (3, 14). The decrease in arginine transport at mid-pregnancy was completely abolished by  $\alpha$ -tocopherol. Such an effect was not observed in virgin rats (Fig. 8).

Finally, to explore the effect of decreased arginine uptake on maternal endothelium, we examined protein nitration using Western blotting and immunohistochemistry utilizing anti-tyrosine antibodies. Figure 9 depicts an electrophoretic profile of nitrotyrosine-modified proteins in aortic rings harvested from pregnant rats. The extent of nitrotyrosine formation was significantly increased in mid-pregnancy compared with virgin animals. Restoration of normal arginine velocities by  $\alpha$ -tocopherol attenuated the increase in protein nitration during mid-pregnancy (Fig. 10).

Immunohistochemical analysis confirmed the aforementioned findings. Figure 11 depicts an augmented staining in mid-pregnancy, predominantly detected in the endothelium, while staining at late pregnancy is minimal. Again, restoration of normal arginine velocities by  $\alpha$ -tocopherol prevented nitrotyrosine staining in aortas harvested from mid-pregnant rats (Fig. 12).

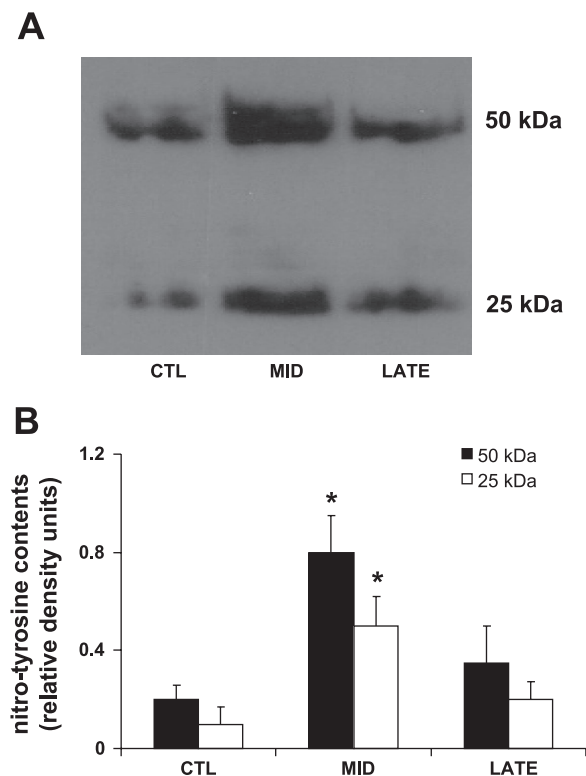


Fig. 9. A: Western blot analysis of nitrotyrosine modified protein expression in aortic rings harvested from virgin and pregnant rats. B: densitometric analysis of nitrotyrosine modified protein contents of the experiments shown in A. Each bar represents the mean of the relative density units  $\pm$  SE from 3 different experiments. \* $P < 0.05$  vs. the corresponding Ctl.

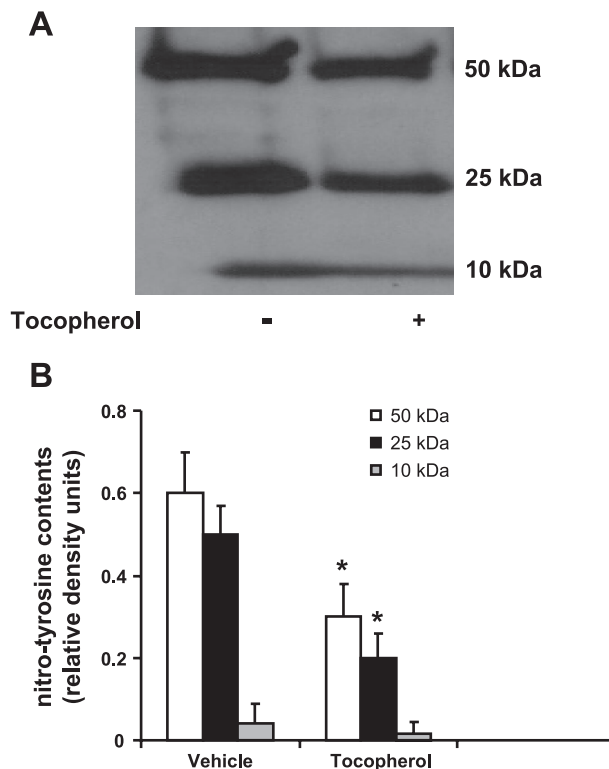


Fig. 10. A: Western blot analysis of nitrotyrosine modified protein expression in aortic rings harvested from pregnant rats, at 12–14 days of pregnancy, treated with either  $\alpha$ -tocopherol or castor oil as a vehicle. These blots are representative of three different experiments. B: densitometric analysis of nitrotyrosine modified protein contents of the experiments shown in A. Each bar represents the mean of the relative density units  $\pm$  SE from 3 different experiments. \* $P < 0.05$  vs. the corresponding vehicle.

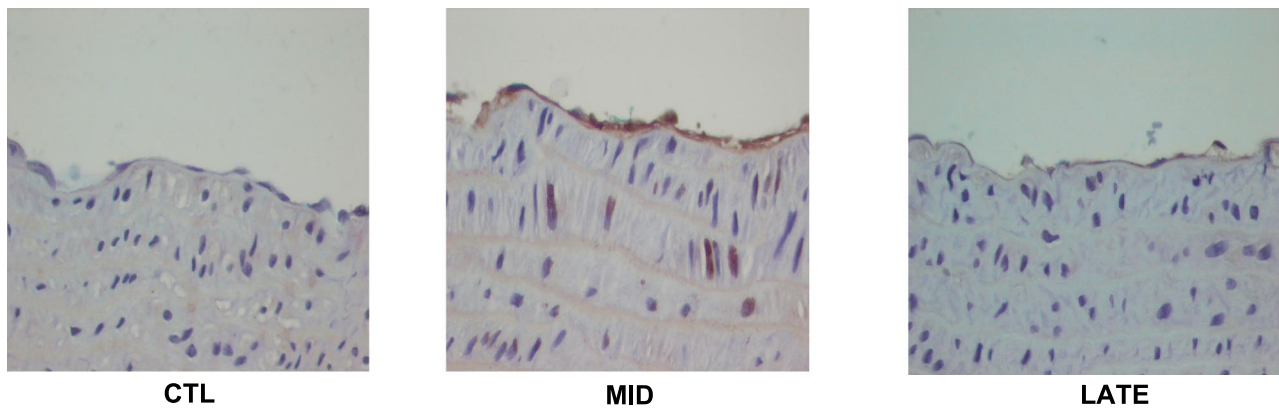


Fig. 11. Immunohistochemistry of aortas, freshly harvested from the various experimental groups stained for nitrotyrosine. A representative slide of one out of three different experiments is shown.

## DISCUSSION

The present study demonstrates that maternal aortic arginine uptake is profoundly attenuated in pregnancy. It is tempting to assume that, rather than supplying its own endothelium, maternal arginine stores are depleted due to a preferential shift to the fetus. The exclusivity of this phenomenon is strengthened by the well-accepted notion that NOS is upregulated in pregnancy (5–7). Normally, arginine transport should match NOS activity. In other words, increase in NO generation is accompanied by augmented arginine transport and vice versa. Our results demonstrate, for the first time to our knowledge, a decrease in arginine transport in a situation in which NO synthesis has been shown to be augmented.

The consequences of decreased arginine transport while NO generation is facilitated are far beyond a mere substrate depletion. NOS enzymes contain four redox active prosthetic groups (FAD, flavin adenine mononucleotide, heme, and tetrahydrobiopterin) that can, in principle, transfer electrons to  $O_2$ . When arginine sources are depleted, a functional NOS may turn into a dysfunctional superoxide-generating enzyme, leading to the accumulation of reactive oxygen species, such as peroxynitrite (29). To test the aforementioned hypothesis, we measured, by using anti-nitrotyrosine antibodies, level of protein nitration, a footprint of oxidation mediated by peroxynitrite (2). Indeed, we found that, during mid-pregnancy, while arginine transport is maximally diminished, protein nitration is significantly intensified. This phenomenon was most predominant in the endothelium and was prevented by restoring normal arginine

uptake velocities by  $\alpha$ -tocopherol administration. These data suggest that, during normal pregnancy, the maternal endothelium is subjected to oxidative stress, possibly resulting from augmented peroxynitrite generation due to “switching” of NOS to a superoxide-generating enzyme in an arginine-depleted environment. Moreover, pregnancy is not simply a state of uncoupling of NOS, but rather a unique and undescribed situation in which both NO and peroxynitrite generation are increased. To what extent this phenomenon affects endothelial function remains elusive. Caution should be taken in interpreting these data, since tocopherol exhibits antioxidant properties that are not directly related to PKC- $\alpha$  inhibition (8, 13). Moreover, the fact that arginine transport was markedly reduced at both mid- and late gestation, while protein nitration was only increased at mid-gestation, suggests that additional factors beside arginine availability play a role in the aforementioned process. Since preeclampsia is a state of acute endothelial dysfunction, one wonders whether these events contribute to the pathogenesis of this serious complication. Our hypothesis implies that diminished arginine transport produces a form of endothelial dysfunction that normally remains asymptomatic. However, in selected individuals, it may evolve to play a role in detrimental hemodynamic alterations, such as preeclampsia. Indeed, restoration of arginine supply has been suggested to minimize the incidence of preeclampsia (9). These findings should be interpreted with caution, since aorta may not pertain to resistance arteries. Therefore, additional studies are needed to determine

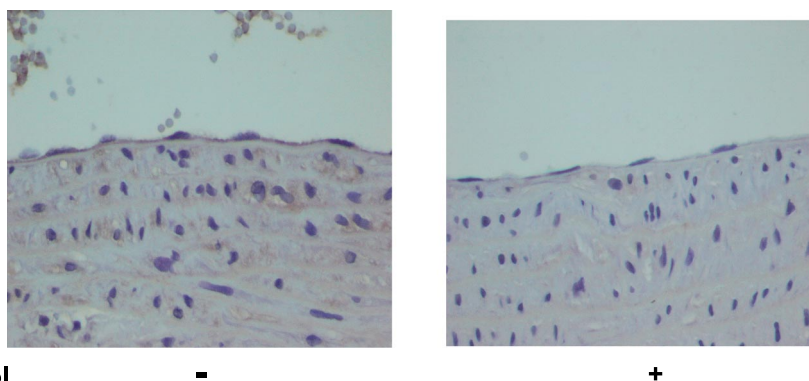


Fig. 12. Nitrotyrosine staining by immunohistochemistry of aortas, freshly harvested from pregnant rats at 12–14 days of pregnancy, treated with either  $\alpha$ -tocopherol (+) or castor oil (–) as a vehicle. A representative slide of one out of three different experiments is shown.

Tocopherol

–

+

whether these events take place in other vascular beds as well.

If these findings were to play a role in the pathogenesis of preeclampsia in selected individuals, they would be expected to present at late rather than mid-pregnancy, when preeclampsia is commonly manifested. Since we were using a rat model to study human pregnancy, apparent differences in the course of maternal hemodynamic changes, which could theoretically relate to arginine metabolism, were reported (21). Studies in humans are required to confirm whether similar changes take place at a different time point during human pregnancy.

We have tried to elucidate a molecular mechanism to explain our findings. The fact that changes in arginine uptake were associated with altered CAT-1 expression supports the notion that these events influence eNOS activity and therefore endothelial function. CAT-1 colocalizes with eNOS in the endothelial cell caveola, thus acting as a specific supplier of arginine for eNOS (18). Our data clearly establish that, in pregnancy, aortic CAT-1 expression is primarily regulated at the posttranslational level. We were intrigued to explore a putative mechanism for the aforementioned phenomenon. A possible involvement of PKC in the regulation of L-arginine transport in different cell types has been discussed for the last several years. There are two lines of evidence suggesting that PKC participates in the regulation of CAT-1 activity. First, according to a model by Albritton and colleagues (1), CAT-1 protein contains three putative sites for phosphorylation by PKC, localized in the fifth and sixth intramolecular loops. Second, both CAT-1 and activated PKC have been reported to be localized in the caveola, allowing for the possibility of CAT-1 and PKC interaction (18, 19).

Graf et al. (11) showed that phorbol myristate acetate, a diacylglycerol analog that directly activates PKC, inhibits CAT-1 transport activity in endothelial cells without reducing CAT-1 mRNA or protein expression. In addition, Krotova et al. (16) found that the classical isoforms of PKC, in particular PKC- $\alpha$ , inhibit CAT-1 transport activity in pulmonary arterial endothelial cells, independent of the expression or intracellular distribution of CAT-1 protein, but rather by modifying its catalytic activity. Our laboratory has recently reported that, in hypercholesterolemic rats, a well-established model of endothelial dysfunction, a posttranslational regulation of CAT-1 was associated with upregulation of aortic PKC- $\alpha$  (26). Indeed, similar to our findings in hyperlipidemia, we found in the present set of experiments a significant increase in aortic PKC- $\alpha$  abundance during pregnancy that was associated with a significant increase in CAT-1 phosphorylation. This finding was previously documented in preeclamptic women and was incriminated in inducing increased endothelial cell permeability (12). Furthermore, when "our" pregnant rats were given  $\alpha$ -tocopherol to inhibit PKC- $\alpha$  activity, the decline in aortic arginine transport was prevented. Taken together, these observations support our hypothesis that upregulation of PKC- $\alpha$  may be the mechanism responsible for diminished arginine transport in pregnancy. This conclusion is somehow weakened by the fact that experiments were performed in whole aortas and PKC- $\alpha$  is also abundant in vascular smooth muscle cells (20).

In conclusion, the present experiments suggest that maternal vasculature is subjected to diminished arginine uptake related to posttranslational inhibition of CAT-1 by PKC- $\alpha$ . These

events are associated with increased protein nitration of the endothelium, reflecting augmented peroxynitrite generation. Further studies are needed to unfold possible deleterious consequences of the aforementioned processes on maternal endothelium during pregnancy.

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