ACE2 expression and activity are enhanced during pregnancy

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The renin-angiotensin system has long been recognized as a critical regulator of blood pressure control in the mammalian organism. ACE2 is a new component of the renin-angiotensin system that has recently been identified (7). Unlike ACE, ACE2 functions as a carboxy-monopeptidase that cleaves a single residue from ANG I, generating ANG 1-9 and a single residue from ANG II to generate ANG 1-7 (32). ANG 1-7 is a biologically active peptide that has vasodilatory properties that counteract the constrictive effect of ANG II (8, 10). ACE2 is thought to modulate, thereby, the effect of the renin-angiotensin system on vascular tone, in parallel to the modulating effect of ACE (9, 25, 36). Our current knowledge of the physiological and pathological significance of ACE2 is incomplete, although it already appears very likely that ACE2 fulfills central regulatory roles in multiple organ systems. ACE2 in pregnancy was the focus of our current investigation.

During normal pregnancy, the renin-angiotensin system is activated. Estrogen and progesterone increase the levels of angiotensinogen and renin, leading, in turn, to a rise in ANG II levels (28). Despite this activation of the renin-angiotensin system, the hemodynamics of normal pregnancy are characterized by a reduction in the systemic vascular resistance (5), no change in the vascular resistance within the utero-placental unit (19), and a lack of rise in systemic blood pressure (20, 28, 29). Systemic arterial pressure even tends to diminish during the second trimester in humans (5), as well as in the third trimester in both normotensive and hypertensive rat models (20, 28, 29). One explanation for the decrease in vascular resistance during pregnancy is that a refractoriness develops to the pressor effect of ANG II (21). Several mechanisms have been proposed to account for this refractoriness, including an effect of prostaglandin or prostaglandin-like agents (11, 13), bradykinin (17), and increased nitric oxide production (33). More recently, it has been demonstrated that the ACE2-ANG 1-7 axis may also be involved in modulating hemodynamics during pregnancy, raising the possibility that ACE2 and ANG 1-7 may account, at least in part, for the resistance to ANG II and the systemic vasodilatation during pregnancy.

Valdes et al. (30) were first to demonstrate in normal pregnant women an increase in urinary ANG 1-7 in parallel to increased urinary ANG II levels throughout gestation. Merrill et al. (22) found that ANG 1-7 levels were also increased in the plasma of women with normal pregnancy. Neves et al. (24) demonstrated increased renal concentration of ANG 1-7 during pregnancy, presumably accounting for increased urinary ANG 1-7. Brosnihan et al. (4) reported enhanced renal immunocytochemical expression of ACE2 and ANG 1-7 during pregnancy. Brosnihan et al. (3) proposed that enhanced ACE2 expression in the kidney counteracts the pregnancy-related elevation in tissue and circulating ANG II. Joyner et al. (15) also reported increased immunocytochemical staining of ANG 1-7 and ACE2 in the kidneys of pregnant rats, demonstrating that the increase was progressive over the course of pregnancy both in the cortex and the medulla. Valdes et al. (31) subsequently tilted the focus from the kidney to the reproductive organs and demonstrated by immunocytochemistry the presence of ANG 1-7 and ACE2 also in the human placenta. Neves et al. (23) pursued these studies in the rat, investigating in

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METHODS

in the control of arterial pressure (6). We carried out our investigation in the Sabra rat model of salt-loading, and/or hypertension affect ACE2 expression andmate of this effect. We also studied ACE2 activity in the uterusall three organs, but providing in addition a quantitative esti-
ons that pregnancy increases expression of ACE2 in hypertensive pregnant rat, aiming to verify thereby previous
sion and activity in the pregnant rat. We evaluated the quan-
titative contribution of the uterus and placenta to ACE2 mRNA and protein levels (6), what is the effect of hypertension during pregnancy on ACE2 expression and activity in the kidneys, uterus, andplacentas?

In the current study, we focused on ACE2 during pregnancy, testing the hypothesis that the reproductive organs, namely, the uterus and placenta, contribute importantly to ACE2 expres-
sion and activity in the pregnant rat. We evaluated the quan-
titative contribution of the uterus and placenta to ACE2 mRNA expression relative to the kidney in the normotensive and hypertensive pregnant rat, aiming to verify thereby previous observations that pregnancy increases expression of ACE2 in all three organs, but providing in addition a quantitative esti-
mate of this effect. We also studied ACE2 activity in the uterus and placenta of the pregnant rat as a functional correlate to ACE2 mRNA expression. Finally, we studied whether strain, salt-loading, and/or hypertension affect ACE2 expression and activity in the kidney, uterus, and placenta during pregnancy. We carried out our investigation in the Sabra rat model of hypertension, in which ACE2 has been previously implicated in the control of arterial pressure (6).

METHODS

Animals

We used for our studies the Sabra hypertension-prone salt-sensitive (SBH/y) and hypertension-resistant salt-resistant (SBN/y) rats from the colony held at the Barzilai Medical Center Campus in Ashkelon, Israel (www.irgc.co.il) (35).

We housed the animals in our center’s animal facility in compliance with institutional regulations and the guidelines set forth by the Israeli Ministry of Health and in accordance with “principles of laboratory animal care” (National Institutes of Health publication no. 85–23, revised 1985) and the guidelines of the American Society of Physiology for the care of laboratory animals. Our experimental protocol was reviewed and approved by the Institutional Ethics Committee for Animal Research. We maintained climate-controlled conditions and set the temperature at 22°C. We kept regular 12-h diurnal light cycles by using an automated light-dark switching device.

Chow

We provided the animals with tap water and standard rat chow containing 0.65% NaCl (Koffolk, Tel-Aviv, Israel), hitherto referred to as regular diet (RD), ad libitum unless stated otherwise. For salt loading, we fed the animals custom prepared diet containing standard chow enriched with 8% NaCl, hitherto referred to as high-salt diet (HSD). In the Sabra rat model, both SBH/y and SBN/y remain normotensive when provided RD; when salt-loaded for 7 wk, SBH/y invariably becomes hypertensive while SBN/y remains normotensive (35).

Study Groups

We studied the animals in the nonpregnant and pregnant state, while feeding them RD or HSD. We investigated, altogether, 8 groups of Sabra rats (SBH/y or SBN/y provided RD or HSD, nonpregnant or pregnant) and two groups of spontaneously hypertensive rats (SHR) provided RD (nonpregnant or pregnant).

Study Protocol

To determine the day of pregnancy during which we would measure ACE2 expression and activity, we initially investigated the blood pressure pattern of nonpregnant and pregnant normotensive and hyper-
tensive rats. We monitored blood pressure in the pregnant animals at predetermined intervals on days 7 and 8 (end of first trimester), days 14 and 15 (end of second trimester), and days 19 and 20 (end of third trimester). We reasoned that if the Sabra rat conforms to reports in other strains, in which a reduction in systemic arterial pressure occurred in the latter part of pregnancy as evidence of a peripheral vasodilatory state (20, 29), we would opt to measure ACE2 expression and activity at that particular time point.

On the basis of the results of our blood pressure measurements (see RESULTS section), we terminated the pregnancies on days 19 and 20 and killed the animals under ether anesthesia. We extracted the uterus, placentas, kidneys, and heart, snap-froze them, and stored them at −70°C until determination of the level of ACE2 mRNA expression and ACE2 enzymatic activity.

In SBH/y, we induced hypertension prior to mating by dietary salt loading. Once hypertension developed, we mated the animals and allowed pregnancy to develop.

Blood Pressure Measurements

We measured systolic blood pressure at ambient temperature (27–28°C) in awake animals by the tail-cuff method, using an IITC photoelectric oscillatory detection device (IITC Life Science, Woodland Hills, CA), as previously described (35). We have previously validated this mode of measurement by telemetry (35).

Expression Analysis

We measured expression of ACE2 at the mRNA level in the kidney, uterus and placenta. For RNA extraction, we used one-quarter of the kidney, including cortex and medulla, the entire uterus, and the whole placenta.

We extracted RNA using the triragent protocol (Molecular Research Center, Cincinnati, OH). To avoid DNA contamination, we used an excess of homogenization solution. To confirm the adequacy of our extraction, we ran 2 μg of the resulting RNA on a 1.5% agarose gel, using 18s and 28s to evaluate quantity and quality. We measured quantitatively ACE2 mRNA abundance in tissue by RT-PCR, using the kit provided by AB-gene (Reverse-It first-strand synthesis kit) and the recommended protocol. In brief, to produce a first-strand cDNA, we used 1 μg total RNA, which we reverse transcribed. We used the first-strand cDNA and amplified ACE2 or GAPDH by PCR with ACE2 or GAPDH-specific primers, as detailed in Table 1. To optimize the conditions of the RT-PCR, we used oligo dT as first-strand
ACE2 IN PREGNANCY

primer, which gave better results than random decamers or hexamers. We determined the required temperature condition to yield one discrete band and tested different numbers of cycles, optimizing the conditions at 35 cycles in the linear phase. The PCR conditions for ACE2 and GAPDH are described in Table 1.

We ran the PCR products on a 1.5% agarose gel and analyzed the resulting bands quantitatively by densitometry using the Labworks computerized system, correcting for GAPDH.

Enzymatic Activity

To measure tissue ACE2 enzymatic activity, we homogenized the tissues in 1XPBS and washed the supernatant twice to lower the level of endogenous phenylalanine. We determined the protein concentration by the Lowry protocol. We determined ACE2 activity using the method described by Huang et al. (14) and modified by Keidar et al. (16). In brief, this method is based on ACE2 cleavage of ANG II and the release of phenylalanine in proportion to ACE2 activity, which in turn, interacts with diaphorase to convert resazurine to fluorescent resorufin, which is measured with a fluorometer (excitation 565 nm, emission 585 nm). We measured fluorescence kinetics over 1 h in a Fluostar Galaxy plate reader (BMG Lab Technologies, Mainz, Germany), using the resulting slope as a measure of ACE2 activity and expressing the data as fluorescence units per minute. To confirm the specificity of the measurements activity to ACE2, we also measured the level of activity in the presence of the specific ACE2 inhibitor GL-0001, formerly known as MLN 4760 (FC 10 M) (generous gift of D. White from GeneLogic, Boston, MA). We calculated net ACE2 activity by subtracting the slope obtained during measurement of total activity from that obtained in the presence of the ACE2 inhibitor, expressing the results in fluorescence units per minute.

Data Presentation and Analysis

ACE2 mRNA abundance and activity are provided for the kidney, uterus, and placenta after correcting for GAPDH and the amount of protein, respectively. Reasoning that during pregnancy an increase in organ size (uterus) and the multiplicity of organs (placentas) affect the total (as opposed to relative) amount of ACE2 expression and activity contributed by each organ system, we are also providing the data after further adjustment for mass and number of organs, as done by others (26, 27). This latter adjustment allowed us to estimate the total contribution of the kidneys, gravid uterus, and placentas to ACE2 expression and activity during pregnancy.

We are providing the data as means ± SE. Statistical analysis included between-group comparisons by the Student’s t-test or one-way ANOVA, as applicable, using the PC-based Statistica software (Statsoft, Tulsa, OK). We set the level of statistical significance at P < 0.05.

RESULTS

Blood Pressure

The blood pressure data are provided in Fig. 1. The salt-sensitive SBH/y but not the salt-resistant SBN/y became hypertensive when fed HSD over 7 wk. When fed RD, there was no rise in BP in either strain. When the animals became pregnant after 7 wk HSD, blood pressure in SBH/y remained elevated and largely unchanged during the first trimester (1st wk) but gradually decreased during the second (2nd wk) and third trimesters (3rd wk). On day 19 of pregnancy, 1 or 2 days prior to delivery, blood pressure in SBH/y on HSD was significantly reduced compared with prepregnancy levels. In SBN/y provided RD and in SBN/y provided HSD or RD, we also observed a much milder yet significant reduction in blood pressure during the third trimester of pregnancy. To verify that the decrease in blood pressure in the latter part of pregnancy was related to pregnancy per se, we continued to measure blood pressure in a select group of animals for 10 days after delivery and found that it returned to prepregnancy values (data not shown). These findings led us to study the expression of ACE2 on days 19 and 20 of pregnancy, 1 or 2 days prior to the expected delivery, during which blood pressure was the lowest in all the strains on all diets, presumably reflective of a systemic vasodilatory state.

Expression of ACE2 at the mRNA Level

ACE2 expression in the uterus and placentas relative to the kidney. To determine the relative level of expression of ACE2 at the mRNA level, we studied the pregnant normotensive SBH/y rat on RD. The level of expression, as expressed per microgram RNA, was highest in the placenta, followed by the kidney and then the uterus, the differences in expression between the three organs being statistically significant (Fig. 2A).

Effect of strain, salt-loading, and hypertension on ACE2 mRNA in the uterus and placentas. To determine the effects of strain, salt-loading, and hypertension on ACE2 expression, we studied SBH/y or SBN/y on RD or HSD. In the uterus of nonpregnant animals, ACE2 mRNA expression was similar in all groups, irrespective of strain, diet, or level of blood pressure; in the uterus of pregnant animals, expression was also similar in all groups (Fig. 3A). In the placenta, ACE2 expres-

Table 1. PCR primers and working protocols for ACE2 and GAPDH

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Variable</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE2</td>
<td></td>
</tr>
<tr>
<td>Forward primer</td>
<td>GTG CAC AAA GGT CAC AAT GG</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>TGT TTC ATC ATG AGG AGG</td>
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<td>Annealing</td>
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<td>Extension</td>
<td>72 C for 1 min</td>
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<td>No. of cycles</td>
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<tr>
<td>Expected band</td>
<td>409 bp</td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
</tr>
<tr>
<td>Forward primer</td>
<td>TCC GCC CCT TCG GCT GAT G</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>CAC GGA AGG CCA TGC CAG TGA</td>
</tr>
<tr>
<td>Annealing</td>
<td>60 C for 30 s</td>
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<tr>
<td>Extension</td>
<td>72 C for 30 s</td>
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<tr>
<td>No. of cycles</td>
<td>30</td>
</tr>
<tr>
<td>Expected band</td>
<td>340 bp</td>
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ACE2, angiotensin-converting enzyme 2.
sion was similar in all groups except in SBH/y fed RD, in which the ACE2 mRNA level was slightly though significantly lower (Fig. 4A).

Effect of pregnancy on ACE2 mRNA expression in the uterus. To determine the effect of pregnancy on ACE2 mRNA expression in the uterus, we compared pregnant to nonpregnant SBN/y and SBH/y rats provided RD or HSD. Pregnancy had an incremental effect on ACE2 mRNA expression within each strain. When expressed per microgram RNA, ACE2 mRNA expression nearly doubled in the gravid uterus of SBN/y on RD or HSD (Fig. 5A and B), only tended to increase in SBH/y on RD (Fig. 5C), and more than doubled in SBH/y rats provided HSD (Fig. 5D).

Since the uterus grows significantly in size during pregnancy, nearly tripling in mass in both SBN/y and SBH/y, irrespective of whether the animals were fed RD or HSD, we reasoned that the total contribution of the gravid uterus to ACE2 mRNA would be better reflected by adjusting the mRNA abundance for the increase in gestational mass of the uterus. This mass adjustment led us to estimate that the placentas contributed the largest amount of ACE2 mRNA, threefold more than the gravid uterus or the kidneys, which contributed equally.

Total contribution of the uterus and placentas relative to the kidneys to ACE2 mRNA during pregnancy. To provide an estimate of the relative total contribution of the uterus, the placentas, and the kidneys to ACE2 mRNA during pregnancy, we calculated the total amount of ACE2 mRNA produced by each organ by correcting for the change in organ mass and the number of organs during pregnancy. For the contribution of the gravid uterus, we multiplied the expression per microgram RNA by the mass. For the placentas, we multiplied expression by the mass of each placenta and then by the average number of placentas per pregnancy, averaging 10 (range 7–13), with no significant differences between SBH/y or SBN/y or whether they were fed RD or HSD. For the kidneys, we multiplied expression by the mass of the kidney during pregnancy and then by 2 for the presence of two kidneys. These calculations led us to estimate that the placentas contributed the largest amount of ACE2 mRNA, threefold more than the gravid uterus or the kidneys, which contributed equally.

Fig. 1. Systolic blood pressure (SBP) in the Sabra rats provided regular diet (RD) or a high-salt diet (HSD) before (weeks 1–7) and during the course of pregnancy (weeks 7–10). ▲ denotes the Sabra hypertension-prone salt-sensitive (SBH/y) HSD (n = 6), ▲ denotes SBN/y HSD (n = 5), and ▼ denotes hypertension-resistant salt-resistant (SBN/y) HSD (n = 5), and ▼ denotes SBN/y RD (n = 4); *P < 0.05, **P < 0.01, compared with blood pressure at the onset of pregnancy (week 7).

Fig. 2. ACE2 mRNA expression (A) in the placenta (n = 4) and uterus (n = 5) shown as a percentage (%) relative to the kidney (n = 5), and activity (B) in the placenta (n = 20) and uterus (n = 21) relative to the kidney (n = 11) in pregnant normotensive SBH/y provided RD; *P < 0.05 compared with the kidney; †P < 0.05 compared with the placenta.
ACE2 Enzymatic Activity in Tissues

To provide a functional measure and correlate to ACE2 expression at the mRNA level, we studied ACE2 enzymatic activity in the uterus, placentas, and kidneys of SBH/y and SBN/y on RD or HSD.

ACE2 activity in the uterus and placenta relative to the kidney. Contrary to our findings with respect to ACE2 mRNA, ACE2 activity per milligram protein in SBH/y-fed RD was highest in the kidney, followed by the placenta and then the uterus (Fig. 2B).

Effect of strain, salt-loading, and hypertension on ACE2 activity in the uterus and placentas. ACE2 activity in the uterus of nonpregnant animals was similar in SBN/y and SBH/y, irrespective of diet, salt-loading or the level of blood pressure (Fig. 3B), in good correlation with mRNA levels. In pregnant animals, ACE2 activity in the uterus was similar in SBN/y and SBH/y on RD, but significantly higher in salt-loaded hypertensive SBH/y group and tended to be higher in salt-loaded nonhypertensive SBN/y (Fig. 3B); such differences were not detected at the mRNA level. In the placenta, ACE2 activity was similar in all groups (Fig. 4B). We did not detect lower ACE2 activity in SBH/y on RD, thus not corroborating the mRNA data in this group.

Effect of pregnancy on ACE2 activity in the uterus. To determine the effect of pregnancy on ACE2 activity in the uterus, we studied pregnant and nonpregnant SBN/y and SBH/y rats provided RD or HSD. When expressed per milligram protein, ACE2 activity in the uterus of SBN/y and SBH/y provided RD was similar in nonpregnant and pregnant animals (Fig. 6, A and C). In SBN/y provided a HSD, pregnancy only tended to increase ACE2 activity compared with the nonpregnant animals (Fig. 6B), whereas in SBH/y on HSD (Fig. 6D), a significant several-fold increase in ACE2 activity was detected. Only this latter finding correlated with our findings at the mRNA level.

Fig. 3. Effect of strain (SBN/y vs. SBH/y), salt-loading (RD vs. HSD) and hypertension (SBH/y on HSD vs. all the rest) on ACE2 mRNA expression (A) and activity (B) in the uterus of nonpregnant and pregnant animals (n = 5–6 within each group); *P < 0.05.

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Fig. 4. The effect of strain (SBN/y vs. SBH/y), salt-loading (RD vs. HSD), and hypertension (SBH/y on HSD vs. all the rest) on ACE2 mRNA expression (A) and activity (B) in the placenta of SBH/y provided RD (n = 5–6 in each group). *P < 0.05 SBH/y RD vs. all other groups.
As with mRNA in the uterus, here, too, we estimated the total contribution of the gravid uterus, as opposed to the nongravid uterus, to ACE2 activity by adjusting ACE2 activity per milligram protein to the increase in gestational mass of uterus. In SBN/y and SBH/y provided RD (Fig. 6, A and C), this adjustment did not alter the results, in opposition to our estimate at the mRNA level. In contrast, in SBN/y and SBH/y fed HSD, this adjustment demonstrated that the total contribution of the gravid uterus to ACE2 activity pregnancy was markedly amplified compared with the nongravid uterus (Fig. 9B), correlating with the total mRNA estimate. Taken together, these findings indicate that in animals provided HSD but not RD, pregnancy is associated with a significant increase in ACE2 activity in the uterus, irrespective of strain. Furthermore, since the increment in activity appeared in both strains and as only salt-loaded SBH/y became hypertensive, the increase in ACE2 activity in the pregnant uterus must be related to salt loading per se and not to hypertension.

**Effect of pregnancy on ACE2 activity in the kidneys.** To determine whether the increase in ACE2 activity observed in the uterus was specific to that organ or part of a generalized multiorgan response to pregnancy, we studied ACE2 activity in the kidneys of pregnant and nonpregnant normotensive SBH/y rats provided RD or of hypertensive SBH/y provided HSD, as we did for ACE2 mRNA. Here, too, pregnancy did not change the level of ACE2 activity in the kidneys of these animals on either diet (data not shown).

**Total contribution of the uterus, placentas, and kidneys to ACE2 activity during pregnancy.** To estimate the total contribution of the uterus, placentas, and kidneys to ACE2 activity in the pregnant animal, we performed calculations similar to those for ACE2 mRNA expression, correcting for the larger mass of the uterus during pregnancy, the presence of multiple placentas, and the presence of two kidneys. These calculations led us to estimate that the total contribution of the placentas to ACE2 activity was the highest, over twofold above that contributed by the two kidneys. The total contribution of the gravid uterus to ACE2 activity was, in contrast to what we had observed for ACE2 mRNA, only one-fifth of that contributed by the kidneys.

**ACE2 enzymatic activity in plasma.** We measured ACE2 activity in 10 μl of serum from 8 pregnant and 9 nonpregnant SBH/y provided a high-salt diet and thus hypertensive. The serum was obtained when the animals were killed at days 19 and 20, i.e., the latter part of the third term of pregnancy, just short of their expected delivery. ACE2 activity in pregnant SBH/y was 1.033 ± 0.066 FU (fluorescence units/min) and in nonpregnant SBH/y 1.213 ± 0.029 FU (P = 0.014 by unpaired t-test). If, however, we assume that pregnant rats are volume expanded by 30–70%, as has been shown by Atherton et al. (1) and Barron (2), the values for serum ACE2 activity in the pregnant rats after correction for the dilution factor would be expected to range from 1.343 to 1.756 FU, higher than those found in nonpregnant SBH/y. In support of a state of volume expansion...
in pregnant SBH/y on a high-salt diet, we measured plasma albumin and found it to be lower in pregnant than in nonpregnant rats, $3.04 \pm 0.10$ g/l ($n = 7$) vs. $3.38 \pm 0.04$ g/l ($n = 9$), respectively, ($P = 0.002$ by unpaired $t$-test).

**DISCUSSION**

In the current study, we confirmed the hypothesis that the reproductive organs, the uterus and the placentas, in addition to the kidneys, contribute importantly to ACE2 expression and activity during pregnancy. We further established that among these three key organs, the overall contribution of the placentas was the foremost, followed in order of importance by the kidneys and the uterus.

We initiated our investigation of the expression of ACE2 at the mRNA level and followed up by setting out to corroborate our findings with ACE2 activity, as a functional correlate of the translational process. We focused specifically on the uterus, which nearly triples in mass during pregnancy, and on the placentas, which constitute de novo but transient organs during pregnancy, a rather unique phenomenon that recurs several times in the life cycle of the mammalian organism. We first successfully validated that ACE2 is expressed at the mRNA levels in both the placenta and the uterus, as reported by Valdes et al. (31) and Brosnihan (3), and that the enzyme exhibits measurable levels of functional activity in both organs. We then compared ACE2 expression and activity in the uterus, the placentas, and the kidneys to obtain a quantitative estimate of their relative importance and contribution. In terms of expression per unit RNA, we found the highest levels of ACE2 mRNA in the placentas, followed by the kidney and then the uterus. In terms of activity per unit mass, however, we found the highest levels of ACE2 activity in the kidney, followed by the placenta and then the uterus. When estimating the total contribution of the uterus and placentas to ACE2 expression and activity, which in our view reflects better the actual in vivo occurrence rather than per unit RNA or mass, we found that the major contributors were the placentas, followed by the kidneys, and the uterus. Interestingly, even though the kidneys and the uterus contributed a similar amount of ACE2 mRNA, the amount of total ACE2 activity contributed by the kidneys was considerably more than by the uterus.

The discordance we observed in mRNA level with functional activity deserves comment. We found in our study such discordance on two occasions: once when we compared mRNA and activity per unit RNA or mass between the placentas and the kidneys and the other time when comparing the total contribution of the uterus and the kidneys to mRNA and activity. The underlying reason may have been technical and organ specific, possibly due to incomplete enzyme extraction and perhaps leading us to underestimate ACE2 activity in these organs. On the other hand, yet undefined posttranscriptional factors may have equally come into play. Regardless, such findings necessarily highlight once again the importance of investigating not only the expression but also its functional...
consequences. In our case, the data suggest that at least in the placentas and uterus, the translation of ACE2 mRNA and/or the activation of the enzyme may be incomplete during pregnancy. However, whether we underestimated ACE2 activity in the reproductive organs or that indeed a posttranscriptional factor was implicated, our findings demonstrate unequivocally that mostly the placentas, but also the pregnant uterus, constitute together important sources of ACE2 mRNA and activity during pregnancy, in addition to ACE2 originating from the kidneys and possibly other sources.

Insofar as the contribution of the kidneys to ACE2 mRNA and activity, our findings failed to confirm previous reports that ACE2 expression is increased in the kidney during pregnancy (3, 4, 15), nor did we find any pregnancy-related increase in ACE2 activity in the kidneys. What is the reason for the discrepancy between our findings and those reported by one other group? Could strain differences account for such disparate findings? We are unclear about this issue, and additional studies in other strains and by other groups might help resolve this matter.

Our data indicate that mostly because of the de novo contribution of the placentas and of the gravid uterus, but not of the kidney, total ACE2 mRNA and activity are likely to have more than doubled during pregnancy compared with the nonpregnant state. Pregnancy constitutes, therefore, a transient physiological condition, in which ACE2 is markedly overexpressed and active. What is the possible significance of ACE2 overexpression during pregnancy and what is its functional role in terms of the mammalian organism? At our current level of understanding, which is clearly incomplete, much of the role of ACE2 is attributed to the generation of the vasodilatory product ANG 1-7. But is the increased activity of ACE2 effective only at the tissue level, generating ANG 1-7 locally as an endocrine effect, or does ACE2 and/or its product, ANG 1-7, also circulate and exert its effects in distal organs, as an autocrine/paracrine effect? There are three possibilities for the action of ACE2: 1) the enzyme could be formed in one organ and act locally by generating ANG 1-7, which is active in situ, 2) ACE2 could lead to the local generation of ANG 1-7, which would, in turn, spill over into the systemic circulation and exert systemic effects, and 3) ACE2, the enzyme, could be synthesized in one organ, shed by ADAM 17 into systemic circulation in its active form (18), reach another organ, and only there generate ANG 1-7 with its resulting effects. In the context of pregnancy, there is evidence that supports all three possibilities. Valdes et al. (31) and Neves et al. (23) support the first possibility by proposing that ACE2 generated in the placenta and uterus is active at the utero-placental interface. Merril et al. (22), who have demonstrated in pregnant women that plasma ANG 1-7 levels are significantly elevated compared with the nonpregnant state, support the second possibility. The third possibility becomes viable when probing the increased urinary ANG 1-7 levels in the urine during pregnancy, which may not be due to increased ACE2 activity in the kidney but rather to increased delivery of ACE2 from the placentas and the gravid uterus to the kidneys. This latter possibility is supported by our findings that ACE2 is indeed shed in its active form and its activity is measurable in the systemic circulation. Such findings lend credibility to an “endocrine” effect of ACE2. Are circulating ACE2 levels, in fact, elevated during pregnancy, a finding that would be consistent with the concept that the placentas and uterus, which generate more ACE2 than in the nonpregnant state, shed this additional amount of ACE2 into the circulation? Our raw measurements of ACE2 activity in serum seemingly suggest that this may not be the case, as we found that serum ACE2 activity in pregnant SBH/y was ~15% lower than in nonpregnant SBH/y. If, however, we consider that in the latter part of pregnancy, plasma volume is increased by 30–70%, then correcting for this increase in plasma volume suggests that ACE2 activity might indeed be increased by 15–55% in the circulation of pregnant vs. nonpregnant animals.

Insofar as assigning a functional role to the increased expression of ACE2 during pregnancy, on the basis of our findings that add up to those of others (3, 23, 31), we consider it likely that ACE2 and ANG 1-7 from the gravid uterus and the placentas, the allegedly “new players on the block” (12), contribute to the resistance to elevated ANG II levels during pregnancy. Thus, ACE2 and ANG 1-7 would prevent impairment of blood flow in the utero-placental unit, the development of systemic hypertension, and possibly account for the gestational reduction in blood pressure. The weakness of our study is that, even though our data are entirely consistent with such effects of ACE2, our conclusions as to the functional role(s) of ACE2 are derived only by association, and our data are insufficient to provide definitive causative proof. Such proof would have been conclusive had we been able to infuse an ACE2 antagonist continuously during pregnancy and had we observed, in the absence of increased ACE2 activity, impairment of blood flow in the utero-placental unity, and/or the development of systemic hypertension, and/or that blood pressure fails to decrease in the latter part of pregnancy. Such critical experiments are pending the availability of an ACE2 antagonist that can be safely and effectively delivered systematically over a prolonged period of time.

Additional results of interest derived from this study are that we did not detect any differences in the level of ACE2 expression or activity in the uterus or the placentas between the two strains that we studied, SBH/y and SBN/y, whether they were in a hypertensive or normotensive state. These findings lead us to conclude that differences in strain or blood pressure do not affect the level of ACE2 expression or activity in the placenta and the uterus. On the other hand, we found that during pregnancy, even though ACE2 expression at the mRNA level is markedly enhanced in the uterus of both strains, irrespective of diet, an increase in ACE2 activity is observed only in the salt-loaded animals. We have no explanation, as yet, as to why the increased message is translated only during salt loading.

**Perspectives and Significance**

If we attempt to extrapolate animal data to humans, our findings indicate that an increase in ACE2 expression and activity may be important for the normal development of pregnancy in both nonhypertensive and hypertensive subjects. Alterations in the expression of ACE2 may lead to abnormalities in the blood pressure pattern during pregnancy and the development of pregnancy-related complications. Should the hypothesis involving ACE2 in the regulation of the utero-placental, as well as in the systemic circulation during pregnancy, be confirmed, then a new therapeutic approach based on modulation of ACE2 expression can be proposed, allowing a
more rational approach to the treatment and possibly prevention of hemodynamically mediated complications of pregnancy.

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