Published in final edited form as:

Placenta. 2007 ; 28(8-9): 878–888.

Regulation of Expression and Localization of the Na⁺/H⁺ Exchanger (NHE) 3 and the NHE Regulatory Factor 2 in Baboon Placental Syncytiotrophoblast by Estrogen

Gerald J. Pepe¹, Marcia G. Burch¹, and Eugene D. Albrecht²

1Department of Physiological Sciences, Eastern Virginia Medical School, Norfolk, VA

2Departments of Obstetrics/Gynecology/Reproductive Sciences and Physiology, The Center for Studies in Reproduction, University of Maryland School of Medicine, Baltimore, MD

Abstract

Our understanding of the regulation of the expression of the sodium hydrogen exchangers (NHE) and their regulatory factors (NHERF), which play important roles in fetal-placental homeostasis, is incomplete. We previously showed that the expression and localization of NHE3 and NHERF2 in the juxtanuclear compartment of the placental syncytiotrophoblast were markedly decreased between mid and late baboon pregnancy. In the current study, immunocytochemical fluorescence localization and level of NHE3/NHE1 and NHERF1/NHERF2 proteins were determined in late gestation in baboons untreated or treated throughout the second half of gestation with an aromatase inhibitor CGS 20267 alone (reduced estrogen levels by >95%) or with estradiol to determine whether estrogen regulated antiporter developmental expression. The immunocytochemical expression of NHE3 and NHERF2 in the juxtanuclear compartment was minimal in baboons untreated or treated with CGS 20267 plus estradiol (i.e. estrogen-replete) but extensive in estrogen-suppressed animals. Moreover, the abundant expression of NHERF2 in fetal vascular endothelium of estrogen-replete baboons was decreased in estrogen-suppressed animals. In contrast, expression and localization of NHE1 and NHERF1 in the placental syncytiotrophoblast were not altered by estrogen deprivation in baboons. Based on our current and previous findings, we propose that estrogen plays an important role in regulating localization and expression of components of the NHE system within and consequently development and function of the primate placental syncytiotrophoblast.

Keywords

Baboon; estrogen; Na⁺/H⁺ exchangers; placenta; pregnancy

INTRODUCTION

The sodium hydrogen exchangers (NHE) and their regulatory factors (NHERF) play an important role in regulating nutrient transport, Na⁺ and H⁺ exchange across the placenta, as well as placental pH, processes important to fetal-placental homeostasis and development

Address Correspondence to: Gerald J. Pepe, Ph.D., Department of Physiological Sciences, Eastern Virginia Medical School, P.O. Box 1980, Norfolk, VA 23501-1980, TEL: (757) 446-5616, FAX: (757) 624-2269, E-Mail: PEPEGJ@EVMS.EDU.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

[1-3]. We previously demonstrated that the subcellular localization of key components of the Na^+/H^+ exchange system in the placental syncytiotrophoblast were altered in association with the increase in estrogen between mid and late baboon gestation [4]. Specifically, the expression of NHE3 and its regulatory factor NHERF2 localized to the trophoblast juxtanuclear compartment at midgestation was markedly decreased near term. In contrast, levels of NHERF2 in an enriched fraction of syncytiotrophoblast basal membrane and fetal vascular endothelium (BM/FVE) exhibited a developmental increase. Moreover, the specific concentrations of NHE1 and its regulatory factor NHERF1, both localized primarily to the microvillus membrane (MVM), were similar at mid and late gestation in association with a 4-fold increase in MVM alkaline phosphatase activity and presumably microvillus surface area as shown in human placenta [5,6]. Despite the physiologic importance of the placental NHE system [3,7-9], our understanding of the factors controlling NHE/NHERF expression and localization in the primate syncytiotrophoblast is incomplete.

Estrogen has been shown to enhance NHERF1 expression in MCF-7 cells [10], whereas cortisol increased NHE3 expression in the adult rat kidney [11] and transcriptional activation of the rat NHE3 gene [12]. Moreover, we previously demonstrated that estrogen regulated the developmental increase in placental syncytiotrophoblast levels and change in the localization of the 11β -hydroxysteroid dehydrogenase-1/-2 (11β -HSD-1/2) enzymes [13,14] controlling glucocorticoid metabolism, biochemical and structural events that we proposed underpinned the increase in placental conversion of cortisol to cortisone that occurs between mid and late gestation [15,16]. Considering these findings, in the current study we utilized our baboon model to determine whether the developmental decrease in NHE3 and NHERF2 expression in the trophoblast juxtanuclear compartment and concomitant increase in NHERF2 in the BM/FVE were regulated by estrogen.

MATERIALS AND METHODS

Animals

Female baboons (*Papio anubis*) weighing 12-18 kg were housed individually in stainless steel cages in air-conditioned quarters and fed Purina monkey chow (Ralston Purina, St. Louis, MO) and fresh fruit and/or vegetables daily and water *ad libitum*. Females were paired with males for five days at the anticipated time of ovulation and pregnancy subsequently confirmed as described previously [17]. Peripheral saphenous blood samples were obtained at 1-4 day intervals during the study period and from the maternal uterine vein at the time of placental-fetal delivery, and serum stored at -20°C until assayed for estradiol levels as described previously [18]. Animals were cared for and used strictly in accordance with USDA regulations and the NIH Guide for the Care and Use of Laboratory Animals (86-23, 1985). The Animal Care and Use Committee of Eastern Virginia Medical School approved the experimental protocol used in this study.

Animal treatment and tissue collection and preparation

Placentas were obtained on day 160-182 of gestation (term = day 184) from baboons untreated (n=5) or treated with the highly specific aromatase inhibitor, CGS 20267 (Letrozole; 4,4-[1,2,3-triazol-1-yl-methylene] bis-benzonitrite, Novartis Pharm AG, Basel Switzerland; 50 $\mu\text{g}/\text{kg}$ body weight/day increasing the dose by 25 $\mu\text{g}/\text{kg}$ at 7-day intervals to a maximum of 115 $\mu\text{g}/\text{kg}$; n=13) or with CGS 20267 plus estradiol benzoate (each at 50-115 $\mu\text{g}/\text{kg}$; n=11) administered sc to the mother beginning on day 100 of gestation as described previously [18]. After removal of the decidua basalis and chorionic plate, 6-7 randomly selected sections of villous placenta were placed in phosphate buffered formalin (Sigma Chemical Co, St. Louis, MO) for immunocytochemistry and 60-70 grams of whole villous tissue used for preparation of fractions of syncytiotrophoblast MVM, cytosol presumably containing endosomes (10,000

× g; 10K), and cytosol containing endosomes and membranes contiguous with the basal membrane (BMm) and the BM/FVE as described previously [4,9]. Although we [4,13] previously showed using these procedures that alkaline phosphatase activity was enriched in the MVM and minimal in other fractions, whereas binding of dihydroalprenolol (DHAP) was enriched in the BM/FVE fraction, activity/binding was also determined in syncytiotrophoblast membrane fractions of animals of the current study.

Immunocytochemistry

Sections (4 μm) of paraffin-embedded placentas were blocked with 5% normal goat serum (NGS; Vector Laboratories, Burlingame, CA) and immunocytochemistry performed as described previously [4]. Briefly, samples were incubated (4°C) overnight with monoclonal antibody to NHE1 (Chemicon International, Temecula, CA), polyclonal antibodies to NHE3 (Chemicon), NHERF1 or NHERF2 (generously supplied by Dr. Chris Yun, Johns Hopkins University, Baltimore, MD) or with a monoclonal antibody to the endosome protein marker, Rab4 (Transduction Laboratories, Lexington, KY) and diluted to 8 μg/ml, 5 μg/ml, 1:7000, 1:1000 or 1:1000, respectively in 5% NGS-PBS. Additional sections were treated prior to incubation with primary antibody with Image iT FX signal enhancer (2 drops/section, as supplied; Molecular Probes Inc., Eugene, OR), blocked with 5% NGS and then treated with streptavidin-biotin blocking reagent (Vector) to further prevent non-specific binding to streptavidin receptors/binding proteins or to endogenous biotin [4]. All sections were then incubated with biotinylated antirabbit or antimouse IgG (Dako Corp., Carpinteria, CA) and then with streptavidin conjugated with AlexaFluor 488 Green (Molecular Probes) diluted 1:500 with 5% NGS. Sections were incubated with Sudan Black (1% in 70% methanol; Sigma) to quench autofluorescence, rinsed and treated (1 min) with propidium iodide (1.0 μg/ml PBS) to stain nuclei red. After application of mounting media (90% glycerol in 0.1 M Tris buffer, pH 9.0 containing 0.0625% n-propyl galate, final concentration), slides were sealed with nail polish and stored in the dark at 4°C until examined using a Zeiss 510 confocal laser microscope (Zeiss Inc., Heidelberg, Germany) or an Olympus BX41 microscope (Optical Elements Corp., Melville, NY) equipped with an Olympus DP70 digital camera with FITC, TRITC and FITC/TRITC filter sets. Under these conditions, expressed proteins were detected as green and those co-localized with nuclei detected as yellow.

Western blot analyses

Western blots were performed as described previously [4,9]. Briefly, after determination of protein concentrations, fractions were loaded (15-25 μg protein/lane) onto discontinuous SDS-polyacrylamide minigels, electrophoresed, wet transferred to Immobilon P (Millipore Corp., Bedford, MA), and membranes blocked with 3% BSA. After incubation (1h) at room temperature with primary antibodies to NHE1 (2.5 μg/ml), NHE3 (5 μg/ml), NHERF1 (1:10,000) or NHERF2 (1:10,000), membranes were treated with goat anti-rabbit or horse anti-mouse IgG horseradish peroxidase-conjugated second antibody (Amersham Life Sciences, Inc., Arlington Heights, IL) and proteins detected using enhanced chemiluminescence (ECL, Amersham). Antibody specificity was confirmed in experiments in which primary antibody was omitted or pre-absorbed with immunizing peptide as described previously [4,9]. To compare levels of protein expression in the syncytiotrophoblast fractions between treatment groups, Western blots were developed in the same film cassette for the respective protein. After film development, samples were quantified by two-dimensional densitometry using an Image 1 analysis system (Universal Imaging Corp., Reamstown, PA) and results expressed as arbitrary densitometric units/μg protein [13].

Na⁺/H⁺ exchange in MVM vesicles

Na⁺/H⁺ activity determined in MVM from placentas of animals treated with CGS 20267 (n=5) or CGS 20267 plus estradiol (n=5) was analyzed concomitantly in MVM of untreated baboons (n=8) as described previously [4]. Briefly, MVM were centrifuged at 15,000 × g, washed with 500 µl ice-cold intravesicular buffer (IVB; pH 6.5) comprised of 25 mM 2[N-morpholino] ethanesulphonic acid (MES; Sigma) and 150 mM KCl, and incubated for 30 min (37°C) in 200 µl IVB containing 10 µM 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein-acetoxymethyl ester (BCECF-AM; Molecular Probes). After washing with IVB, samples were incubated for 15 min (4°C) in 600 µl IVB without/with amiloride (Sigma) at a concentration (0.5 mM) shown to inhibit NHE activity by >98% [7]. MVM vesicles (200 µg protein/600 µl IVB) were then placed into one chamber of a KinTek SF 2001 Stopped Flow spectrofluorimeter (KinTek Laboratories, La Marque, TX) and 2 ml of extravesicular buffer (EVB; pH 9.5) comprised of 10 mM HEPES (Sigma) and 150 mM NaCl added to a second chamber. After equilibration to 20°C, 60 µl of MVM (20 µg protein) was mixed instantaneously with 150 µl EVB, which increased extravesicular pH to 8.2, and voltage (i.e. fluorescence; excitation 490 nm; emission 535 nm) recorded (6 data points/sec) for 30 sec. At the conclusion of the reaction, sample was displaced and another 60 µl of MVM and 150 µl EVB were mixed and the analyses repeated. Thus, for each MVM sample, at least 10 determinations of NHE activity in presence/absence of amiloride were performed with the computer software determining a best fit two-exponential of the average of 6 of the analyses as well as amplitude (A2), adjusted for differences in loading, and rate constant (k2) from which t_{1/2} (k2 = 0.7/t_{1/2}) and Na⁺/H⁺ activity (A2 × k2) expressed as millivolts (mV)/sec/20 µg protein were calculated. We previously confirmed that leakage of dye from vesicles was minimal (< 2%) over a 60 min period and that comparable amount of dye were released by treatment with 1% Triton.

Statistics

Data are expressed as an overall mean (± SE) and analyzed by unpaired “t” test or analysis of variance with post hoc comparisons of the means by the Newman-Keuls multiple comparison tests. Because fetal/placental weights, Na⁺/H⁺ activity in MVM as well as the levels of serum estradiol and the antiporters and their regulatory factors in syncytiotrophoblast fractions determined by Western blot in untreated baboons of the current study were comparable to respective values previously reported in a group of contemporaneously studied untreated baboons [4], respective values were combined for statistical purposes. Data on alkaline phosphatase and Na⁺/H⁺ activity in MVM as well as DHAP binding in BM/FVE from placentas of untreated baboons was determined concomitantly with that in animals treated with CGS 20267 ± estrogen and previously published [4].

RESULTS

Serum estradiol and placental and fetal weight

Maternal peripheral serum estradiol levels increased from approximately 1 ng/ml on days 85-120 of gestation to 1.5 ng/ml to 2.5 ng/ml on days 120-150 and to 3.0 - 3.5 ng/ml by day 165-175. Within 48-72 h of the onset of CGS 20267 treatment on day 100, maternal serum estradiol levels decreased to and remained at approximately 0.1 ng/ml, whereas the pattern of serum estradiol in baboons treated with CGS 20267 plus estradiol benzoate was similar to that in untreated animals. Thus, mean (± SE) estradiol levels in uterine venous serum on the day of delivery in untreated baboons (Table 1) were decreased (P<0.05) by more than 95% in animals treated with CGS 20267 and restored (P<0.05) to approximately 55% of normal by treatment with CGS 20267 and estrogen. The day of delivery was similar in baboons untreated or treated with CGS 20267 ± estradiol.

Although fetal body weight in late gestation was not altered by treatment with CGS 20267, placental weight was increased ($P<0.05$) by approximately 40%. As a consequence, in estrogen-suppressed baboons, the ratio of fetal body weight to placental weight was lower ($P<0.05$) than in untreated animals. The fetal-placental weight ratio was restored to normal in baboons treated with CGS 20267 and estrogen, however placental and fetal weights were 22% and 14% lower ($P<0.05$) than respective values in untreated baboons (Table 1).

Placental alkaline phosphatase activity and DHAP binding

Alkaline phosphatase activity was comparably enriched in MVM isolated from placentas of baboons treated with CGS 20267 (12 ± 3 fold) and CGS 20267 $\pm$ estrogen (12 ± 1 fold) and not significantly different from that noted in placental MVM from untreated baboons (19 ± 4 fold).

DHAP binding in BM/FVE was also comparably enriched in baboons treated with CGS 20267 (30 ± 6 fold) or CGS 20267 and estrogen (28 ± 3 fold) and similar to that in BM/FVE isolated from placentas of untreated baboons (35 ± 3 fold).

Of the trophoblast fractions examined, protein levels in the BMm (11-14 mg/g placental weight) were greater than in the 10K (0.9-1.2 mg/g), the MVM (0.2-0.4 mg/g) and BM/FVE (0.13–0.14 mg/g). Protein concentrations in respective syncytiotrophoblast fractions were similar in baboons treated with CGS 20267 or CGS 20267 plus estradiol and comparable to values in placental fractions isolated from untreated baboons.

Expression of NHE3 and NHERF2

In untreated baboons, NHE3 protein was abundantly expressed (Fig. 1 A, and inserts 1 and 2) and detected as green in syncytiotrophoblast cytoplasm presumably associated with endosomes, and to a lesser extent in FVE of the inner villous core. Although NHE3 was detected in the juxtanuclear compartment, expression at this site was minimal and not observed in all sections. Thus, the majority of syncytiotrophoblast nuclei remained dark, i.e. devoid of green protein (Fig 1A, insert 1) or red (Fig 1A and insert 2) indicating that co-localization of expressed green protein with propidium iodide around syncytiotrophoblast nuclei was minimal. In contrast, in placentas of estrogen-suppressed baboons (Fig 1B and inserts 1 and 2), although NHE3 was detected in the FVE and remained abundantly expressed in and localized to syncytiotrophoblast cytoplasm, the antiporter was also detected in the juxtanuclear compartment in the majority but not all regions of the syncytium. Thus, in most instances, syncytiotrophoblast nuclei appeared green (Fig 1B, insert 1) or yellow (Fig 1B and insert 2). Moreover, the absence of yellow dots in the nucleolus (Fig 1B, insert 2) indicates that NHE3 was localized around but not within syncytiotrophoblast nuclei. Overall, the pattern and site of NHE3 expression in placentas of baboons treated with CGS 20267 and estrogen approached that observed in untreated baboons. Thus, in estrogen-treated baboons, in the majority of sections examined, NHE3 was minimally detected in the juxtanuclear compartment (Fig. 1 C, inserts 1 and 2), whereas expression in the cytoplasm (Fig. 1 C and insert 1) remained relatively abundant. Specificity was confirmed by absence of staining in sections incubated without primary antibody.

Presence of endosomes in syncytiotrophoblast of untreated baboons was confirmed by detection of the endosome specific marker Rab4 which was abundantly expressed in the cytoplasm but not the nucleus (data not shown). Expression of Rab 4 in syncytiotrophoblast cytoplasm was not altered by CGS 20267 or CGS 20267 plus estrogen treatment. Specificity was confirmed by absence of staining in sections incubated without primary antibody.

NHE3 was detected by Western blot (Fig. 2) as an 85 kDa protein in the BMm and 10K, cytoplasmic fractions presumably containing endosomes, but was not detected in the MVM (not shown). Thus, consistent with the cytoplasmic expression of NHE3 detected by immunocytochemistry, the mean levels (arbitrary units $\times 10^{-3}/\mu\text{g}$ protein) of NHE3 in the BMm and 10K fractions were similar ($P>0.60$) in baboons untreated (BMm, 42 ± 4 ; 10K, 50 ± 4) or treated with CGS 20267 (BMm, 43 ± 4 ; 10K, 54 ± 3) or CGS 20267 and estrogen (BMm, 36 ± 3 ; 10K, 45 ± 3).

As with NHE3, in untreated baboons NHERF2 protein was also abundantly expressed in and localized to the syncytiotrophoblast cytoplasm as well as the FVE, but appeared to be relatively absent from the juxtannuclear compartment (Fig. 3A, and inserts 1 and 2). Thus, the majority of syncytiotrophoblast nuclei were devoid of green protein and therefore remained dark (Fig. 3A, insert 1) or red (Fig 3A and insert 2). However, in estrogen-suppressed baboons (Fig. 3B and inserts 1 and 2) although NHERF2 expression remained abundant in the FVE and in syncytiotrophoblast cytoplasm, NHERF2 was also detected in the juxtannuclear compartment in several but not all regions of the syncytium. Thus, in the majority of sections syncytiotrophoblast nuclei appeared green (Fig. 3B, insert 1) or yellow (Fig 3B, and insert 2) indicative of NHERF2 protein expression whereas the nucleolus when visible appeared red, i.e. devoid of NHERF2 protein (Fig. 3B, insert 2). In contrast, in baboons treated with CGS 20267 and estrogen, NHERF2 was minimally detected in the juxtannuclear compartment (Fig. 3C and inserts 1 and 2), whereas expression in the cytoplasm (Fig. 3C, inserts 1 and 2) and FVE remained relatively abundant. Finally, in virtually all sections of placenta examined, NHERF2 expression although abundant in FVE was not detected in other cells of the inner villous core or in the MVM in baboons untreated or treated with CGS 20267 $\pm$ estrogen (Fig 3 A, B and C).

Western blot (Fig. 4) showed that NHERF2 protein was detected as doublet with an approximate molecular size of 45 kDa in the BM/FVE of baboons untreated or treated with CGS 20267 $\pm$ estradiol. However, the mean level (arbitrary units $\times 10^{-3}/\mu\text{g}$ protein) of NHERF2 protein in BM/FVE of untreated baboons (41 ± 3) was reduced by approximately 65% ($P<0.05$) in CGS 20267-treated animals (27 ± 4) and restored ($P<0.05$) to normal in animals treated with CGS 20267 plus estrogen (45 ± 4).

Expression of NHE1 and NHERF1

As seen in Fig 5, NHE1 was abundantly expressed in syncytiotrophoblast MVM of untreated baboons (Fig. 5A) and expression at this site did not appear to be altered by treatment with CGS 20267 (Fig. 5B) or CGS 20267 and estrogen (Fig. 5C). In all animals, NHE1 expression was negligible in syncytiotrophoblast nuclei, minimal in the syncytial cytoplasm, and lightly but only occasionally detected in FVE. Consistent with immunocytochemistry, NHE1 was detected by Western blot (Fig. 5D) as a 104 kDa protein and levels (arbitrary units $\times 10^{-3}/\mu\text{g}$ protein) in the MVM in untreated baboons (36 ± 4) were not altered by treatment with CGS 20267 (36 ± 1) or CGS 20267 and estrogen (34 ± 5).

The expression of NHERF1 was also abundantly expressed and detected in syncytiotrophoblast MVM, but was not detected in nuclei, and only weakly expressed in cytoplasm and FVE. NHERF1 expression in the MVM appeared similar in baboons untreated (Fig. 5E) or treated with CGS 20267 (Fig. 5F) or CGS 20267 and estrogen (Fig. 5G). Consistent with the immunocytochemistry, mean level (arbitrary units $\times 10^{-3}/\mu\text{g}$ protein) of the 50 kDa NHERF1 protein in the MVM of baboons treated with CGS 20267 (39 ± 5) was similar to those in baboons untreated (42 ± 5) or treated with CGS 20267 and estrogen (45 ± 5 ; Fig. 5H).

Na⁺/H⁺ Exchange Activity

Fig. 6 depicts representative time-course of Na⁺/H⁺ exchange activity of MVM vesicles prepared from placentas of baboons treated with CGS 20267 (A) or CGS 20267 plus estradiol (B). The original and computer generated 2-exponential fit of the average of 6 experimental determinations of change in fluorescence (i.e. voltage) of BCECF loaded MVM vesicles incubated for 30 sec with buffer which raised pH from 6.9 to 8.2 and extravesicular NaCl from 0 to 150 mM are depicted. In all MVM vesicles there was a rapid ($t_{1/2} < 2$ sec) initial period of Na⁺/H⁺ exchange which was followed by a relatively more slow ($t_{1/2} = 15$ to 20 sec) increase in exchange activity which was maintained for up to 30 sec. The latter Na⁺-dependent component of Na⁺/H⁺ exchange (A2; k2) reflected coupled activity of an NHE antiporter in the MVM and not uncoupled H⁺ or Na⁺ fluxes as only this component Na⁺/H⁺ exchange (i.e. change in fluorescence) was inhibited >98% in vesicles incubated with 0.5 mM amiloride (not shown). Moreover, fluorescence did not change when BCECF loaded vesicles were incubated with intravesicular buffer (low pH, contains no sodium) to which NaCl was omitted (Fig 6A, 6B). Similar results were obtained in MVM from placentas of untreated baboons [not shown; 4]. Thus, as summarized in Fig 6C, mean Na⁺/H⁺ exchange activity (2.55 ± 0.31 mV) and $t_{1/2}$ (18.4 ± 0.8 sec) of Na⁺-dependent Na⁺/H⁺ exchange of MVM vesicles of baboons treated with CGS 20267 were similar to respective values in MVM vesicles of animals untreated (3.02 ± 0.12 mV; 16.2 ± 0.8 sec) or treated with CGS 20267 plus estradiol (2.94 ± 0.24 ; 19.9 ± 1.5 sec).

DISCUSSION

The results of the current study demonstrate that the decline in expression of NHE3 and its regulatory factor NHERF2 in the syncytiotrophoblast juxtanuclear compartment that occurs between mid and late baboon gestation [4] was prevented in animals in which estrogen production was suppressed during this interval of pregnancy. Moreover, the change in NHE3 and NHERF2 localization was restored to normal by treatment with CGS 20267 and estrogen. Therefore, we propose that the reduction in expression of NHE3 and NHERF2 in the juxtanuclear compartment of the primate placenta with advancing gestation is an estrogen-dependent event. Estrogen, via interaction with estrogen receptor α (ER α), has been shown to enhance the mRNA levels of the endosomal marker Rab 11 and thus perhaps endosome formation in uterine endometrial cell cultures [19] and we have shown that the baboon placenta expresses ER [20]. However, whether the detection of NHE3 in the juxtanuclear compartment and presumably endosomes of the placenta in CGS 20267-treated baboons as well as the decline in NHE3 at this site between mid and late gestation [4] reflects a change in the amount of NHE3 in and/or the number of endosomes that localize to the nuclear envelope remains to be determined. It is also well established that expression of NHE3 protein, activation of NHE3 gene expression and protein production as well as NHE3 activity are up-regulated by cortisol [11,12]. We previously showed that estrogen increased the ratio of 11 β -HSD2 to 11 β -HSD1 in the BM of baboon syncytiotrophoblast between mid and late gestation [13,14]. Because 11 β -HSD2 catalyzes the conversion of cortisol to biologically inactive cortisone [21 for review], we proposed that this estrogen-dependent switch in enzyme ratio in syncytiotrophoblast may act to control local levels of active cortisol that arrive within the fetal-placental unit. Therefore, whether estrogen controls NHE3/NHERF2 expression in the juxtanuclear membrane directly and/or indirectly via enhancing the conversion of cortisol to cortisone remains to be determined.

Using immortalized kidney cell lines, others have shown that NHE3, which is abundantly expressed in and localized to endosomes in the cytoplasm and in the juxtanuclear compartment, functions at these sites in part to acidify endosomes and to facilitate vesicular fusion [22,23]. Moreover, it has been proposed that the trafficking of NHE3 to/from the cell membrane and

the juxtanuclear compartment [24] represents a physiologic mechanism by which NHE3 controls Na^+/H^+ exchange in epithelial cells of the kidney, colon, small intestine and gallbladder [25,26]. It also appears that trafficking of NHE3 can be enhanced by NHERF2, apparently via activation of phospholipase C [26]. Although the specific physiologic role(s) of NHE3 and NHERF2 in syncytiotrophoblast intracellular function remains to be elucidated, based on our studies we propose that estrogen plays an important role in regulating expression of components of the NHE system within and consequently development and function of the primate placental syncytiotrophoblast.

In contrast to the decrease in NHERF2 expression in the juxtanuclear compartment, protein levels of this regulatory factor in the cytoplasm remained unchanged whereas expression in the FVE was increased during the second half of pregnancy [4]. In animals of the current study, although NHERF2 levels in syncytiotrophoblast cytoplasm were not altered by estrogen, levels in the BM/FVE were reduced in estrogen-suppressed baboons and restored to normal in animals treated with CGS 20267 and estrogen. Therefore, although it is likely that estrogen may exert a direct effect on NHERF2 expression in FVE, the latter remains to be established since we have also shown that vessel density was increased between mid and late gestation in the baboon [28]. Finally, the apparent differences in NHERF2 expression in syncytiotrophoblast and fetal vascular cells may also reflect other developmental changes in the placental:fetal hormonal milieu. For example, local levels of cortisol markedly decrease in the placenta with advancing gestation whereas levels in the fetus are increased as a result of onset of cortisol production by the fetal adrenal gland [29].

It is well established that NHE1 functions primarily to maintain cell volume and intracellular pH [1,30]. The results of the current study showed that suppression of estrogen production in baboons was associated with an increase in placental wet weight and a decrease in the fetal:placental weight ratio. It is possible that the increase in placental weight in estrogen-suppressed animals may reflect an increase in accumulation of fluid/water and perhaps cellular volume secondary to changes in expression and localization of components of the NHE system. Although NHE3/NHERF2 expression/localization were altered by CGS 20267 administration, levels of NHE1 and its regulatory factor NHERF1 in the MVM, a major site of Na^+/H^+ exchange between the maternal and trophoctodermal compartments, as well as NHE activity were not altered in estrogen-suppressed baboons. However, because NHE1 expression and activity is rapidly up-regulated by alterations in cell volume and/or intracellular pH [31], it is possible that the apparent maintenance of MVM NHE1 expression/activity in estrogen-suppressed baboons reflects upregulation secondary to changes in placental intracellular H^+ , volume and/or osmolality. It is also possible that in addition to level of expression, interaction of these antiporters with other integral components of the MVM may have been altered by estrogen deprivation. For example, formation of microvilli is dependent upon phosphorylation of ezrin as well as NHERF1 to anchor F-actin to plasma membrane proteins including NHE1 or NHE3 [32-35]. Thus, formation of MVM and Na^+/H^+ exchange appear to be linked although the latter remains to be confirmed for the primate placenta.

In summary, the results of the current study show that the decline in expression of and localization of NHE3 and NHERF2 in the juxtanuclear compartment of syncytiotrophoblast of the baboon placenta during the second half of baboon pregnancy was prevented in animals in which estrogen production was suppressed by administration of an aromatase inhibitor. Moreover, the changes in NHE3 and NHERF2 localization were restored to normal by treatment with aromatase inhibitor and estrogen. Based on our current and previous findings, we propose that estrogen plays an important role in regulating expression of components of the NHE system within and consequently development and function of the primate placental syncytiotrophoblast.

Acknowledgements

The authors greatly appreciate the generous provision of CGS 20267 by Novartis Pharma AG, Basel Switzerland. The authors sincerely appreciate the secretarial assistance of Ms. Sandra Huband with the manuscript and preparation of the photomicrographs. The generous supply of NHERF1 and NHERF2 antibodies provided by Dr. Chris Yun, John Hopkins University, Baltimore, MD is greatly appreciated.

*This work was supported by National Institutes of Health Research Grant R01 HD-13294.

References

1. Orlowski J, Kandasamy RA, Shull GE. Molecular cloning of putative members of the Na/H exchanger gene family. *J Biol Chem* 1998;267:9331–9339. [PubMed: 1577762]
2. Mahendran D, Donnai P, Glazier JD, D'Souza SW, Boyd RD, Sibley CP. Amino acid (system A) transporter activity in microvillous membrane vesicles from the placentas of appropriate and small for gestational age babies. *Pediatr Res* 1993;34:661–665. [PubMed: 8284106]
3. Sibley CP, Glazier JD, Greenwood SL, Lacey H, Mynett K, Speake P, Jansson T, Johansson M, Powell TL. Regulation of placental transfer: the Na⁺/H⁺ exchanger-a review. *Placenta* 2002;23:S39–S46. [PubMed: 11978058]
4. Pepe GJ, Burch MG, Albrecht ED. Developmental regulation of the Na⁺/H⁺ exchangers and their regulatory factors in baboon placental syncytiotrophoblast. *Endocrinology* 2006;147:2986–2996. [PubMed: 16527850]
5. Ludwig, H. 1974 Surface structure of the human placenta. In: Moghissi, KS.; Hafez, ES., editors. *The Placenta, Biological and Clinical Aspects*. Springfield: CC Thomas; 1974. p. 40-64.
6. Benirschke K. Anatomical relationship between fetus and mother. *Ann NY Acad Sci* 1994;731:9–20. [PubMed: 7944139]
7. Hughes JL, Doughty IM, Glazier JD, Powell TL, Jansson T, D'Souza SW, Sibley CP. Activity and expression of the Na⁺/H⁺ exchanger in the microvillous plasma membrane of the syncytiotrophoblast in relation to gestation and small for gestational age birth. *Pediatr Res* 2000;48:652–659. [PubMed: 11044487]
8. Johansson M, Glazier JD, Sibley CP, Jansson T, Powell TL. Activity and protein expression of the Na⁺/H⁺ exchanger is reduced in syncytiotrophoblast microvillous plasma membranes isolated from preterm intrauterine growth restriction pregnancies. *J Clin Endocrinol Metab* 2002;87:5686–5694. [PubMed: 12466372]
9. Pepe GJ, Burch MG, Sibley CP, Davies WA, Albrecht ED. Expression of the mRNAs and proteins for the Na⁺/H⁺ exchangers and their regulatory factors in baboon and human placental syncytiotrophoblast. *Endocrinology* 2001;142:3685–3692. [PubMed: 11459818]
10. Ediger TR, Kraus WL, Weinman EJ, Katzenellenbogen BS. Estrogen receptor regulation of the Na⁺/H⁺ exchanger regulatory factor. *Endocrinology* 1999;140:2976–2982. [PubMed: 10385389]
11. Baum M, Moe OW, Gentry DL, Alpern RJ. Effect of glucocorticoids on renal cortical NHE-3 and NHE-1 mRNA. *Am J Physiol* 1994;267:F437–F442. [PubMed: 8092257]
12. Kandasamy RA, Orlowski J. Genomic organization and glucocorticoid transcriptional activation of the rat Na⁺/H⁺ exchanger NHE3 gene. *J Biol Chem* 1996;271:10551–10559. [PubMed: 8631855]
13. Pepe GJ, Burch MG, Albrecht ED. Localization and developmental regulation of 11 β -hydroxysteroid dehydrogenase-1 and -2 in the baboon syncytiotrophoblast. *Endocrinology* 2001;142:68–80. [PubMed: 11145568]
14. Pepe GJ, Burch MG, Albrecht ED. Estrogen regulates 11 β -hydroxysteroid dehydrogenase -1 and -2 localization in placental syncytiotrophoblast in the second half of primate pregnancy. *Endocrinology* 2001;142:4496–4503. [PubMed: 11564715]
15. Pepe GJ, Albrecht ED. Transuteroplacental metabolism of cortisol and cortisone during mid- and late gestation in the baboon. *Endocrinology* 1984;115:1946–1951. [PubMed: 6489265]
16. Pepe GJ, Waddell BJ, Stahl SJ, Albrecht ED. The regulation of transplacental cortisol-cortisone metabolism by estrogen in pregnant baboons. *Endocrinology* 1988;122:78–83. [PubMed: 3335215]
17. Albrecht ED. A role for estrogen in progesterone production during baboon pregnancy. *Am J Obstet/ Gynecol* 1980;136:569–574.

18. Albrecht ED, Aberdeen GW, Pepe GJ. The role of estrogen in the maintenance of primate pregnancy. *Am J Obstet Gynecol* 2000;182:432–438. [PubMed: 10694348]
19. Chen D, Genapathy P, Zhu LJ, Xu X, Li Q, Bagchi IC, Bagchi MK. Potential regulation of membrane trafficking by estrogen receptor α via induction of Rab11 in uterine glands during implantation. *Mol Endocrinol* 1999;13:993–1994. [PubMed: 10379897]
20. Pepe GJ, Davies WA, Dong K, Luo H, Albrecht ED. Cloning of the 11β -hydroxy-steroid dehydrogenase (11β -HSD) -2 gene in the baboon: effects of estradiol on promoter activity of 11β -HSD-1 and -2 in placental JEG-3 cells. *Biochim Biophys Acta* 1999;1444:101–110. [PubMed: 9931459]
21. Pepe GJ, Albrecht ED. Actions of placental and fetal adrenal steroid hormones in primate pregnancy. *Endocrine Rev* 1995;16:608–648. [PubMed: 8529574]
22. Gekle M, Drumm K, Mildenerberger S, Freudinger R, Gassner B, Silbernagl S. Inhibition of Na^+ - H^+ exchange impairs receptor-mediated albumin endocytosis in renal proximal tubule-derived epithelial cells from opossum. *J Physiol* 1999;520(Pt 3):709–721. [PubMed: 10545138]
23. Gekle M, Freudinger R, Mildenerberger S. Inhibition of Na^+ - H^+ exchanger-3 interferes with apical receptor-mediated endocytosis via vesicle fusion. *J Physiol* 2001;531:619–629. [PubMed: 11251045]
24. Janecki AJ, Montrose MH, Zimniak P, Zweibaum A, Tse CM, Khurana S, Donowitz M. Subcellular redistribution is involved in acute regulation of the brush border Na^+/H^+ exchanger isoform 3 in human colon adenocarcinoma cell line Caco-2. *J Biol Chem* 1998;273:8790–8798. [PubMed: 9535857]
25. D'Souza S, Garcia-Cabado A, Yu F, Teter K, Lukacs G, Skorecki K, Moore HP, Orlowski J, Grinstein S. The epithelial sodium-hydrogen antiporter Na^+/H^+ exchanger 3 accumulates and is functional in recycling endosomes. *J Biol Chem* 1998;273:2035–2043. [PubMed: 9442041]
26. Akhter S, Kovbasnjuk O, Li X, Cavet M, Noel J, Arpin M, Hubbard AL, Donowitz M. Na^+/H^+ exchanger 3 is in large complexes in the center of the apical surface of proximal tubule-derived OK cells. *Am J Physiol Cell Physiol* 2002;283:C927–C940. [PubMed: 12176749]
27. Choi JW, Lee-Kwon W, Jeon ES, Kang YJ, Kawano K, Kim HS, Suh PG, Donowitz M, Kim JH. Lysophosphatidic acid induces exocytic trafficking of Na^+/H^+ exchanger 3 by E3KARP-dependent activation of phospholipase C. *Biochim Biophys Acta* 2004;1683:59–68. [PubMed: 15238220]
28. Hildebrandt VA, Babischkin JS, Koos RD, Pepe GJ, Albrecht ED. Developmental regulation of vascular endothelial growth/permeability factor messenger ribonucleic acid levels in and vascularization of the villous placenta during baboon pregnancy. *Endocrinology* 2001;142:2050–2057. [PubMed: 11316772]
29. Pepe GJ, Albrecht ED. Regulation of the primate fetal adrenal cortex. *Endocrine Rev* 1990;11:151–176. [PubMed: 2180686]
30. Sardet C, Franchi A, Pouyssegur J. Molecular cloning, primary structure, and expression of the human growth factor-activatable Na^+/H^+ antiporter. *Cell* 1989;56:271–280. [PubMed: 2536298]
31. Noël J, Pouyssegur J. Hormonal regulation, pharmacology, and membrane sorting of vertebrate Na^+/H^+ exchanger isoforms. *Am J Physiol (Cell Physiol)* 1995;268:C283–C296.
32. Bretscher A, Reczek D, Berryman M. Ezrin: a protein requiring conformational activation to link microfilaments to the plasma membrane in the assembly of cell surface structures. *J Cell Sci* 1997;110:3011–3018. [PubMed: 9365271]
33. Sato N, Funayama N, Nagafuchi A, Yonemura S, Tsukita S, Tsukita S. A gene family consisting of ezrin, radixin and moesin. Its specific localization at actin filament/plasma membrane association sites. *J Cell Sci* 1992;103:131–143. [PubMed: 1429901]
34. Arpin M, Algrain M, Louvard D. Membrane-actin microfilament connections: an increasing diversity of players related to band 4.1. *Curr Opin Cell Biol* 1994;6:136–141. [PubMed: 8167019]
35. Yun CH, Oh S, Zizak M, Steplock D, Tsao S, Tse CM, Weinman EJ, Donowitz M. cAMP-mediated inhibition of the endothelial brush border Na^+/H^+ exchanger, NHE3, requires an associated regulatory protein. *Proc Natl Acad Sci (USA)* 1997;94:3010–3015. [PubMed: 9096337]

Fig 1.

Representative photomicrographs of the immunocytochemical expression of NHE3 in placental syncytiotrophoblast on day 165 of gestation (term = day 184) in baboons untreated (A and inserts 1 and 2) or treated on days 100-164 with CGS 20267 (B and inserts 1 and 2) or CGS 20267 and estradiol benzoate (C and inserts 1 and 2) as described in legend to Table 1. Sections were incubated with antibodies to NHE3, stained with streptavidin conjugated with AlexaFluor 488 and treated with propidium iodide to stain nuclei red. Proteins localized to cytoplasm or syncytiotrophoblast membranes were detected by immunofluorescence as green and those colocalized to juxtannuclear compartment stained as yellow. Original magnification

= ×400 A-C; inserts enlarged approximately 2-fold. FVE = fetal vascular endothelium; cyto = cytoplasm; nl = nucleolus.

Fig 2.

Representative Western immunoblot (top Panels) and mean ($\pm$ SE) concentrations (arbitrary units/ μ g protein) of the 85 kDa NHE3 protein in the BMm (A) and 10K (B) fractions prepared from placentas obtained on day 165 of gestation from baboons untreated (BMm, $n = 7$; 10K, $n = 9$) or treated with CGS 20267 (BMm, $n = 7$; 10K, $n = 7$) or CGS 20267 and estradiol benzoate (BMm, $n = 7$; 10K, $n = 6$). Proteins were loaded (15-25 μ g/lane) onto discontinuous SDS polyacrylamide gels, transferred to Immobilon P and incubated with primary antibody to human NHE3. Mean ($\pm$ SE) values of NHE3 in the BMm and 10K fractions in treatment groups were not significantly different ($P > 0.6$; ANOVA).

Fig 3.

Representative photomicrographs of the immunocytochemical expression of NHERF2 in placental syncytiotrophoblast on day 165 of gestation (term = day 184) in baboons untreated (A, and inserts 1 and 2) or treated on days 100-164 with CGS 20267 (B, and inserts 1 and 2) or CGS 20267 and estradiol benzoate (C, and inserts 1 and 2). Sections were incubated with antibodies to NHERF2, stained with streptavidin conjugated with AlexaFluor 488 and treated with propidium iodide to stain nuclei red. Proteins localized to cytoplasm or syncytiotrophoblast membranes were detected by immunofluorescence microscopy as green and those colocalized to juxtannuclear compartment stained as orange-yellow. Original

magnification = $\times 400$ A-C; inserts enlarged approximately 2-fold. FVE = fetal vascular endothelium; cyto = cytoplasm; nl = nucleolus.

Fig 4.

Representative Western immunoblot and mean ($\pm$ SE) concentrations (arbitrary units/ μ g protein) of the 45 kDa NHERF2 protein in syncytiotrophoblast basal membrane/fetal vascular endothelium (BM/FVE) isolated from placentas obtained on day 165 of gestation from baboons untreated ($n = 10$) or treated with CGS 20267 ($n = 9$) or CGS 20267 + estradiol ($n = 8$) and analyzed as described in legend to Fig 2. Values with different letter superscripts differ from each other at $P < 0.05$ (ANOVA; Student Newman-Keuls multiple statistic).

Fig 5.

Representative photomicrographs of the immunocytochemical expression of NHE1 (A-C) and NHERF1 (E-G) in baboon placental syncytiotrophoblast on day 165 of gestation in animals untreated (A, E) or treated with CGS 20267 (B, F) or CGS 20267 and estradiol benzoate (C, G). Sections incubated with primary antibody to NHE1 or NHERF1, stained and analyzed as described in legend to Fig 1. Original magnification = $\times 400$. Representative Western immunoblot and mean ($\pm$ SE) concentrations of the 104 kDa NHE1 (D) and NHERF1 (H) protein in syncytiotrophoblast MVM isolated from placentas obtained on day 165 of gestation from baboons untreated (NHE1, $n=10$; NHERF1, $n=7$) or treated with CGS 20267 (NHE1, $n=9$; NHERF1, $n=8$) or CGS 20267 and estradiol (NHE1, $n=6$; NHERF1, $n=8$).

Fig 6.

A,B; Representative time-course of Na⁺/H⁺ exchange activity of MVM vesicles prepared from placentas of baboons treated with CGS 20267 (A) or CGS 20267 plus estrogen (B). Exponential fit ($Y = A_1(-k_1t) + A_2(-k_2t) + C$) of change in fluorescence (i.e. voltage) of BCECF loaded MVM vesicles incubated for 30 sec with buffer without NaCl or buffer which raised extravesicular NaCl to 150 mM. A1; k1, initial period of exchange; A2; k2, Na⁺-dependent Na⁺/H⁺ exchange C: Overall mean (± SE) Na⁺/H⁺ exchange activity (mV/sec/20 μg protein) of MVM vesicles isolated from placentas of baboons untreated or treated with CGS 20267 and CGS 20267 plus estrogen and calculated as the product of the rate constant and amplitude of the second exponential (k2 and A2). T½ = 0.7/k2.

Placental and fetal body weights, uterine venous serum estradiol (E₂) levels in and day of delivery of baboons untreated or treated with the aromatase inhibitor CGS 20267.

Table 1

Treatment	Day of Delivery	Uterine E ₂ (ng/ml)	Placenta (g)	Fetal Body Weight (g)	Ratio Fetal/Placental Weight
Untreated	170 ± 2.1	5.9 ± 0.9 ^a	183 ± 8 ^a	831 ± 29 ^a	4.6 ± 0.2 ^a
CGS 20267	165 ± 1.4	0.4 ± 0.1 ^b	221 ± 8 ^b	832 ± 25 ^a	3.8 ± 0.2 ^b
CGS 20267 + estradiol	166 ± 1.5	3.1 ± 0.6 ^c	142 ± 5 ^c	720 ± 33 ^b	4.9 ± 0.2 ^a

Uterine blood samples, fetuses and placentas obtained on day 160-182 of gestation from baboons untreated or treated with CGS 20267 (50-115 µg/kg BW/d) or CGS 20267 and estradiol benzoate (50-115 µg/kg BW/d each) administered sc to the mother beginning on day 100 (term = day 184). Values (mean ± SE; n = 10-13/group) with different letter superscripts differ from each other at P<0.05 (ANOVA; multiple comparison of the means using the Student-Newman-Keuls statistic).