Tetrahydrobiopterin preferentially stimulates activity and promotes subunit aggregation of membrane-bound calcium-dependent nitric oxide synthase in human placenta

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Type III nitric oxide synthase (NOS III) is responsible for >90% of nitric oxide (NO) synthesizing activity in first trimester placentae. Enzyme activity is distributed between cytosolic (30%) and membrane-bound forms (70%), with highest specific activity observed in microsomal fractions. In the present study, the effect of tetrahydrobiopterin (BH₄) on subunit structure and activity of microsomal and cytosolic NOS III was compared.

As revealed by immunoblot analysis, incubation of microsomal membranes with 50 µM final concentration BH₄ for 10 min at 37°C resulted in a striking conversion of monomeric NOS III into a protein having the characteristics (electrophoretic mobility, resistance to sodium dodecyl sulphate) of the homodimeric form. In contrast, BH₄ induced significantly less marked changes in the NOS III dimer content of cytosolic fractions. Enzyme activity in microsomes is stimulated ~6-fold upon addition of 50 µM BH₄, while only a 2-fold activation is detectable in cytosolic fractions. Taken together, the observations suggest that BH₄ activates NOS III in the primordial human placenta by promoting its subunit assembly in the membrane, while cytosolic NOS III is relatively insensitive to BH₄. Compartment-specific action of BH₄ represents a novel mechanism which is implicated in the regulation of placental NO activity.

Key words: dimerization/endothelial nitric oxide synthase/primordial placenta (human)/ tetrahydrobiopterin

Introduction

Nitric oxide (NO) has emerged as an important signal and effector molecule in a variety of mammalian tissues, including the reproductive systems (Lowenstein et al., 1994). NO is generated together with L-citrulline from L-arginine and O₂ by a family of enzymes called NO synthases (NOS; E.C.1.14.13.39.). So far three different isofoms have been isolated, cloned and characterized (for recent reviews see Forstermann et al., 1994; Nathan and Xie, 1994; Sessa, 1994; Griffith and Stuehr, 1995; Wang and Marsden, 1995). Type I NOS (NOS I) was first identified in neurons, Type II (NOS II) in macrophages and Type III (NOS III) in endothelial cells. NOS I and III are constitutively expressed in different tissues and are activated by cytoplasmic Ca²⁺ transients. In contrast, NOS II is active at resting intracellular Ca²⁺ concentrations, and synthesis of this isofom is inducible in a variety of cell types. NOS enzymes are thought to be active as homodimers, and require five different co-factors: haeme, NADPH, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and tetrahydrobiopterin (BH₄).

Although the exact role(s) of BH₄ in NO synthesis is yet to be elucidated, available evidence points to an allosteric activation rather than electron donor function. BH₄ enables arginine to bind to NOS I (Klatt et al., 1994) and it was shown to stabilize the axial ligand geometry of the NOS haeme iron in NOS I and NOS II (Wang et al., 1995). BH₄ protects NOS I from NO inactivation (Griscavage et al., 1994), and it is essential for formation of stable active NOS I and NOS II homodimers in purified enzyme preparations (Hevel et al., 1991; Stuehr et al., 1991; Schmidt et al., 1991; Baek et al., 1993; Klatt et al., 1995) or in cells (Tzeng et al., 1995). Dimerization is obligatory for NO synthesis by NOS II, and the same mechanistic model seems to apply for NOS I as well (Klatt et al., 1995). Interestingly, in contrast to the increasing body of data on the role of BH₄ in NOS I and NOS II function, very little is known concerning its role in the mechanism of action of NOS III. While several reports have shown that addition of BH₄ to purified NOS III preparations, cell or tissue homogenates results in various degrees of stimulation (Werner-Felmayer et al., 1993; Rosenkranz-Weiss et al., 1994; Schoedon et al., 1994; Chen et al., 1995), detailed characterization of the phenomenon has never been accomplished. Recently, BH₄-induced activation was reported to have minimal effect on dimerization of bovine NOS III expressed in Escherichia coli (Rodriguez-Crespo et al., 1996).

Trophoblast cells of first trimester placentae express high amounts of NOS activity, 30% of which is found in the cytosol and ~70% is localized to intracellular membranes, with highest specific activity observed in microsomal fractions (Kukor and Tóth, 1994; Tóth et al., 1995). A similar enzyme distribution has also been reported for term placentae (Conrad et al., 1993). More than 90% of placental NOS activity is strictly calcium-dependent and, based on its biochemical and immunological characteristics, appears to be identical with the endothelial type (NOS III) isofom (Conrad et al., 1993; Myatt et al., 1993; Buttery et al., 1994). Levels of trophoblastic nitric oxide activity are highest in the first trimester during human
pregnancy and much higher than those in the myometrium (Ramsey et al., 1996) and in the oviduct (Rosselli et al., 1996). Therefore, first trimester placenta provide an excellent model system for studies on the biochemical properties, physiological role and regulation of NOS III. In addition, such studies may shed some light on the role of NOS activity in normal and pathological placental physiology (Morris et al., 1996).

Recently, we have shown that addition of exogenous BH₄ to fresh homogenates stimulates NOS activity 2–2.5-fold, with half-maximal stimulation observed at a final BH₄ concentration of 26 µM (Kukor et al., 1996). Importantly, BH₄ had no effect on the calcium dependency of the enzyme, the apparent Km values for Ca²⁺ being comparable in the presence or absence of BH₄. The results indicate that BH₄ is present in limiting concentrations and raise the possibility that it may act as an important regulator of NO synthesis in placental tissue. In the present study, in an attempt to gain insight into the mechanism of BH₄ action on NOS III in the human placenta, the effect of BH₄ on the subunit structure and catalytic activity of microsomal and cytosolic NOS III was compared. The observations suggest that BH₄ potently activates membrane-bound NOS III through promoting its subunit aggregation, while the cytosolic enzyme is relatively insensitive to this co-factor.

Materials and methods

Materials

L-[2,3,4,5-³H]-Arginine-HCl (64 Ci/mmol, 2.37 µg/Bq/mmol) was purchased from Amersham International (Chalfont, Bucks, UK). (6R)-5,6,7,8-Tetrahydro-L-biopterin-2HCl (BH₄) was obtained from Research Biochemicals International (Natick, MA, USA). Polyclonal NOS III antiserum and endothelial cell lysate were from Transduction Laboratories (Lexington, KY, USA). Horseserialid peroxidase-conjugated anti-rabbit IgG, N³-nitro-arginine methylster (NAME), and Dowex 50X8-400 were from Sigma Chemical Co. (St. Louis, MO, USA).

Tissue, homogenization and fractionation

Primordial human placentae were obtained from legal instrumental interruption of 8–10 week old pregnancies at the 2nd Department of Obstetrics and Gynecology, Semmelweis University of Medicine, Budapest, Hungary. Use of the tissue for these experiments was approved by the Ethics Committee of the Medical Section of The Hungarian Academy of Sciences. Placentae were homogenized in 2 volumes of ice cold homogenizing solution containing 40 mM HEPES-Na pH 7.4, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 µg/ml leupeptin, 10 µg/ml soybean trypsin inhibitor and 0.2 µg/ml aprotinin. The homogenate was filtered through a nylon mesh and heavy particulate material and mitochondria were sedimented at 15 000 g for 30 min in a Beckman J-21 centrifuge. The supernatant was centrifuged at 100 000 g for 60 min in a Beckman L2–75B ultracentrifuge to obtain microsomal pellet and cytosolic fraction.

Immunoblotting

Cytosolic and microsomal fractions were solubilized in 2× electrophoresis sample buffer containing 4% sodium dodecyl sulphate (SDS) and 200 mM DTT, final concentrations of 2% and 100 mM respectively. Samples were frozen overnight, thawed on ice and electrophoresed on 7.5% SDS–polyacrylamide gels. Electrophoresis was performed at 29 mA for 2 h using the MiniProtein II setup (Bio-Rad) placed in an ice-bath. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon, Millipore) for 2 h at 290 mA, and membranes were blocked overnight at 4°C with TBST solution (20 mM Tris–HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20) containing 5% dissolved milk powder. Polyclonal NOS III antiserum was used at a dilution of 1:400 at room temperature for 2 h. Peroxidase-conjugated secondary antibody [goat anti-rabbit immunoglobulin (Ig)G] was used at 1:10 000 dilution for 1 h at room temperature. Signals were developed with a 1 min incubation with chemiluminescent substrate (ECL) and exposed to X-ray films for 15–30 s. Developed films were scanned using an Ultrascan XL Laser Densitometer (LKB, Bromma, Sweden).

Measurement of NO-synthase activity

A total of 100 µl of microsomal or cytosolic enzyme preparation (0.5–2.5 mg protein) was incubated with 0.4 mM NADPH, 1 mM citrulline, 1 mM MgCl₂, 16 mM t-valine, 20 mM HEPES-Na pH 7.4, 12 IU/ml calmodulin, 0.75 µCi [³H]-arginine (46.9 µCi/µg) in 250 µl final volume for 10 min at 37°C. Incubated cytosolic and microsomal fractions contained final arginine concentrations of 75 ± 2 µM and 25 ± 7 µM (mean ± SD) respectively, which is well above the Kₘ (1.8 µM) reported for the placental NOS enzyme (Conrad et al., 1993). We have previously reported the endogenous BH₄ content of early human placenta (207 pmol/g wet tissue, Kukor et al., 1996). Based on this value, both the microsomal and cytosolic incubates can be regarded as BH₄-depleted, since calculated BH₄ concentrations were in the nanomolar range (25 nM or less) where no significant effect on NOS activity was detectable (M.Tóth, unpublished results). Calcium-dependent NOS (cNOS) activity was assayed in the presence of 25 µM free Ca²⁺ concentration, adjusted with Ca²⁺-EGTA buffer ( Bártfai, 1979). Calcium-independent enzyme (ciNOS) activity was measured in the presence of 1 mM EGTA. In control incubates, 1 mM NAME and 1 mM EGTA were included in the assay mixture. All assays were run in triplicates. Incubations were initiated by addition of the enzyme preparation and terminated by adding 2 ml ice-cold stop solution containing 20 mM HEPES-Na pH 5.0, 2 mM EDTA, and 1 mM NAME. The terminated incubates were centrifuged at 1000 g for 5 min. The supernatants were loaded onto 0.8×1.5 cm columns of DOWEX 50X–8–400 cation exchange resin (Na⁺ cycle) and [³H]-citrulline was eluted with 2 ml distilled water. The flow-through (4.25 ml) was collected, and radioactivity was counted as previously described (Kukor and Tóth, 1994; Tóth et al., 1995).

In order to obtain cNOS catalysed [³H]-citrulline formation, radioactivities recovered from assays performed with Ca²⁺ were corrected for the c.p.m. obtained with 1 mM EGTA. To determine cNOS-catalysed [³H]-citrulline formation, radioactivity value obtained from assays containing 1 mM EGTA and NAME was subtracted from the c.p.m. values measured in the presence of 1 mM EGTA. Total NOS activity (i.e. cNOS + ciNOS) was measured in the presence of 25 µM Ca²⁺ and the resulting c.p.m. values were corrected for incubation with 1 mM NAME + 1 mM EGTA. C.p.m. values recovered in [³H]-citrulline were converted to pmol citrulline values using an average arginine concentration of 542 nmol/g wet placenta tissue and 79.3 and 1.24% distribution of [³H]-arginine in cytosol and microsomal fractions (M.Tóth, B.Asbóth and Z.Kukor, unpublished data). NO synthase activity was expressed finally as pmole citrulline/min/mg protein.

Protein determinations

Protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as standard.

M.Sahin-Tóth et al.
Nitric oxide synthesis in human placenta

Figure 1. Panel A: Comparison of type III nitric oxide synthase (NOS III) immunoreactivity in endothelial cell lysate (E) and microsomes from first trimester placenta (P). 6 µg of commercial endothelial cell lysate (Transduction Laboratories) and 35 µg microsomal membranes were subjected to immunoblot analysis as described in the legend to Panels B and C and in the Materials and methods section. Panel B: Effect of sample-boiling and tetrahydrobiopterin (BH4) on the subunit structure of microsomal NOS III of the placenta. Panel C: Effect of sample-boiling and BH4 on the subunit structure of cytosolic NOS III of the placenta. Microsomal membranes or cytosolic fractions were incubated in the absence or presence of 50 µM BH4, for 10 min at 37°C, in complete incubation medium except radioactive arginine. Subsequently, 2× electrophoresis sample buffer containing 200 mM dithiothreitol (DTT) and 4% sodium dodecyl sulphate (SDS) was added and where indicated samples were boiled for 2 min or kept on ice. Approximately 40 µg microsomal (Panel B) or cytosolic (Panel C) protein were subjected to SDS–polyacrylamide gel electrophoresis and electroblotted. The polyvinylidene difluoride (PVDF) membranes were incubated with NOS III-specific antiserum. After incubation with the secondary antibody (anti-rabbit goat immunoglobulin G) coupled to horseradish peroxidase, followed by a short incubation with chemiluminescent substrate, the membranes were exposed to film for 30 s.

Results

Tetrahydrobiopterin induces dimerization of membrane-linked NOS III

It has been demonstrated for NOS I and NOS II that BH4 promotes assembly of active homodimers from inactive monomeric NOS. To test the possibility that a similar phenomenon occurs with NOS III, microsomal membrane fractions of placenta were subjected to immunological analysis using NOS III specific antiserum. As shown in Figure 1, Panel A, if samples are boiled for 2 min in electrophoresis sample buffer containing 2% SDS and 100 mM DTT before electrophoresis, the immunoreactive band of microsomal NOS III migrates at ~140 kDa, and it is indistinguishable from NOS III produced in endothelial cells. Using these sample preparation conditions, incubation of microsomal membranes with 50 µM BH4 has no effect on the migration of the immunoreactive band (Figure 1, Panel B). In contrast, as recently shown for NOS I (Klatt et al., 1995), if high temperature treatment of samples is avoided and electrophoresis is performed at low temperatures (see Materials and methods section for details), NOS III with higher order subunit structure becomes detectable on Western blots (Figure 1, Panel B).

Panel B of Figure 1 clearly demonstrates that a 10 min incubation with 50 µM BH4 converts monomeric NOS III to an apparently 210 kDa immunoreactive form. Based on similar results with NOS I, II, and III, we identified this form as NOS III homodimers (Klatt et al., 1995; Tzeng et al., 1995; Rodriguez-Crespo et al., 1996). This is supported by the observation that homodimers composed from purified bovine NOS III (Rodriguez-Crespo et al., 1996) exhibited on SDS–PAGE gels an apparent molar mass (206 kDa) which is essentially identical to that found here.

Different preparations of microsomes contained variable amounts of dimer before BH4 treatment, possibly due to slight variations in endogenous BH4 concentrations. Importantly, in every microsomal preparation tested, addition of BH4 significantly increases the amount of dimer, and densitometric measurements regularly revealed a parallel decrease in the monomeric NOS III complement (data not shown). Interestingly, intact native membrane structure did not appear to be a requirement for dimerization, since incubation of microsomal fractions solubilized in electrophoresis sample buffer with 50 µM BH4 also stimulated conversion of monomeric NOS to dimers (not shown). This observation also indicates that, similarly to NOS I (Klatt et al., 1995), dimerization of NOS III represents an unusually tight interaction, and it readily occurs under strong denaturing conditions. In contrast to microsomes, BH4 has a much less marked effect on NOS III found in cytosolic fractions of early placentae (Figure 1, Panel C, also Figure 3). It should be noted that, when sample boiling is omitted, due to increased non-specific immunological background on Western blots, monomeric NOS may be difficult to visualize (Figures 1 and 3). Importantly, the 210 kDa region is clear of background, and no significant increase in the specific immunoreactivity is detected here when cytosolic fractions are incubated in the presence of 50 µM BH4 and boiling is omitted, compared with boiling omission alone.
Figure 2. Effect of tetrahydrobiopterin (BH4) on the total (Ca2+-dependent plus Ca2+-independent) nitric oxide synthase (NOS) activity (A) and on the Ca2+-dependent (cNOS) and Ca2+-independent (ciNOS) NOS activities (B) of placental microsomal and cytosolic fractions. Microsomal (0.5–2.5 mg) or cytosolic (1.5–2.0 mg) protein was incubated in the standard reaction mixture at 37°C for 10 min and the formation of [3H]-citrulline from [3H]-arginine was determined. Total NOS activity = enzyme activity measured with microsomes or cytosol in the presence of 25 μM Ca2+ using NAME + EGTA to correct for background. cNOS activity = enzyme activity measured with microsomes or cytosol in the presence of 25 μM Ca2+ using EGTA to correct for background. ciNOS activity = enzyme activity measured with microsomes or cytosol with no Ca2+ added and in the presence of 1 mM EGTA, using NAME to correct for background. The c.p.m. values have been converted to pmole citrulline as described in the Materials and methods section. Combined results (mean ± SEM) from four experiments with triplicate incubations each are presented.

Tetrahydrobiopterin potently and preferentially enhances activity of membrane-bound NOS III

The differences observed in the effect of BH4 on dimerization of microsomal and cytosolic NOS III raise the possibility that BH4 may affect enzyme activity in a different fashion in these subcellular compartments. Figure 2 demonstrates the combined results of four experiments, in which changes in NOS enzyme activity were assessed. When total NOS activity in microsomal and cytosolic fractions was assayed in the presence of 25 μM Ca2+, addition of 50 μM BH4 stimulated membrane-bound NOS nearly 4-fold, while only a 1.7-fold stimulation was observed with soluble NOS (Figure 2A). Because early placental exhibit both Ca2+-dependent (cNOS) and Ca2+-independent (ciNOS) activities (Kukor and Tóth, 1994; Tóth et al., 1995), the stimulatory effect may have resulted from interaction with either of these subpopulations. However, when the effect of BH4 was tested in the presence of 1 mM EGTA (Figure 2B), only insignificant activation (microsomes) or no activation at all (cytosol) was detected, confirming previous observations that activity of the calcium-independent NOS (ciNOS) is not affected by BH4 (Kukor et al., 1996). Remarkably, in contrast, the Ca2+-dependent NOS population (cNOS, i.e. NOS III) was stimulated about 6-fold in microsomes, while only a 2-fold stimulation was observed in the cytosol (Figure 4B). While the stimulatory effect of BH4 on enzyme activity was strictly dependent on the presence of calcium ions, BH4-promoted subunit assembly of NOS III did not require elevated calcium concentrations. When the effect of BH4 on subunit structure was assessed in the presence of 1 mM EGTA (Figure 3), similar results were obtained as seen in Figure 1, Panels B and C. BH4-induced conversion of monomers to dimers was strong in the microsomal fraction, while a much less marked effect was observed in the cytosol. The observations suggest that dimerization may be necessary but not sufficient for activation of NO production.

Discussion

In the study presented here the effect of BH4 on microsomal (i.e. membrane-bound) and cytosolic (i.e. soluble) NOS III in human placenta is compared. Our previous observations have shown that addition of exogenous BH4 to fresh homogenates of first trimester placentae leads to a 2–3-fold stimulation of NOS activity, indicating that BH4 may act as an important regulator of NO synthesis in this tissue (Kukor et al., 1996). Since enzyme activity is distributed between membrane-bound and cytosolic forms (Kukor and Tóth, 1994; Tóth et al., 1995), it raises the possibility that the two enzyme pools may be differently regulated by BH4. In support of this, it has been described that in freshly isolated pig endothelial cells the two NOS III fractions have different catalytic and regulatory properties (Hecker et al., 1994). Available evidence from studies on NOS I and NOS II indicates that BH4 exerts its effect through promotion of dimerization of NOS subunits with consequent activation of the enzyme. Therefore, we
initially investigated whether or not BH₄ promotes a similar subunit aggregation of placental NOS III. Immunological analysis of microsomal and cytosolic fractions by Western blotting leads to two important conclusions: (i) BH₄ induced the formation of SDS-resistant aggregates of NOS III, with a molar mass (210 kDa) which is indistinguishable from that reported for bovine NOS III (206 kDa, Rodriguez-Crespo et al., 1996) indicating that all three NOS isoforms respond to this co-factor in an analogous fashion; (ii) BH₄ induced a high degree of subunit assembly in the microsomes, while soluble NOS was much less affected. The observations suggest a compartment-specific action of BH₄, where the membrane-bound isofrom is the primary target of the co-factor. Subsequently, the effect of BH₄ on the catalytic activity of NOS III in the two subcellular fractions was compared. Remarkably, 50 µM BH₄ stimulated calcium-dependent NOS activity in placental microsomes nearly 6-fold, while a much less pronounced (approximately 2-fold) activation was observed in cytosolic fractions. Calcium-independent enzyme activity in either fraction was not affected by BH₄, confirming previous observations that this enzyme pool is not subject to BH₄ regulation (Kukor et al., 1996). Taken together, the results suggest that BH₄ activates membrane-bound NOS III, possibly through promoting its subunit assembly in the membrane, while cytosolic NOS III is relatively insensitive to BH₄.

On the basis of recent reports and observations presented here, it appears that BH₄-promoted dimerization and activation are common features of all three NOS isoforms. Compartment-specific action of BH₄, however, represents a novel mechanism whereby NOS III may be regulated. Several other regulatory pathways have been described, and a complex picture of enzyme regulation is unfolding in which calcium transient-activated NO output is controlled by at least two mechanisms triggered by hormones or cytokines: (i) translocation of NOS from the particulate to the soluble fraction alters the soluble to membrane-bound ratio of NOS III in the cell. The two fractions may have different enzymatic and regulatory properties, including drastically different sensitivity to BH₄; (ii) elevation of BH₄ concentrations selectively enhances calcium-stimulatable activity of membrane-anchored enzyme, while cytosolic NOS III is much less affected. Obviously, the extent of BH₄ activation is primarily dependent on the size of the membrane-bound enzyme pool. In addition, other regulatory mechanisms, including phosphorylation (Davda et al., 1994; Ohara et al., 1995) or intracellular alkalinization (Fleming et al., 1994) may contribute to the overall NOS activity. It is possible that these additional mechanisms exert their effect through NOS III distribution or BH₄ activation.

Clearly, one of the most intriguing findings of the present studies is the apparent compartment specificity of BH₄. Several possible explanations exist for the phenomenon, leading to new, testable models of enzyme regulation and maturation in human placenta and other NOS III-expressing tissues. Binding of NOS III to membranes may result in higher local enzyme concentrations, facilitating subunit aggregation and enzyme activation. In addition, membranes may serve as surface catalysts for the BH₄-induced reactions, and those may proceed according to zero-order reaction kinetics, i.e. in a concentration-independent manner. Alternatively, the ratio of monomeric and dimeric forms in the cytosolic and microsomal enzyme pools may differ. As a consequence, in microsomes, where monomeric forms appear to be abundant, BH₄-induced subunit aggregation and enzyme activation is strong. In contrast, in the cytosol, where active dimers may predominate, BH₄ effect is minimal.

A question that must be addressed is the physiological significance of these effects which were measured using 50 µM BH₄, whereas the concentration of this co-factor in the placental tissue is ~200 nM (Kukor et al., 1996). In a separate study using placental microsomes, we have demonstrated a half-maximal ‘dimerizing activity’ at 148 nM BH₄ and a nearly maximal effect at ~1 µM BH₄. Furthermore, both effects were attended by a proportionate increase of NOS activity (Tóth et al., 1997). These data suggest that the subunit aggregating effect of BH₄ is a crucial physiological mechanism for the assembly of active Ca²⁺-dependent NOS in the primordial human placenta.

Finally, it has not escaped our attention that regulation of NOS III activity by BH₄ and promotion of dimerization may have an impact on the normal and abnormal functioning of the placenta as well as other parts of the pregnant uterus. Thus, we speculate that a decrease in the BH₄ concentration may be related to a reported decrease of NO production immediately before delivery (Natuzzi et al., 1993; Sladek et al., 1993), which may contribute to increased sensitivity of myometrium to contractile agonists, such as prostaglandins and oxytocin. On the other hand, a role for BH₄ in the increased production of NO in the uterine cervix at term, with consequent promotion of cervical extensibility (Buhimschi et al., 1996), is also possible. Furthermore, it is attractive to postulate that hypertension in pregnancy and pre-eclampsia could be the result of impaired placental BH₄ formation with an attending decrease of NO output (Morris et al., 1996). These speculations may stimulate further studies on this enzyme activating mechanism and on its role in the reproductive functions of the uterus in general and in the placenta in particular.

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