Estrogen Stimulates von Willebrand Factor Production by Cultured Endothelial Cells

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Monolayers of cultured human umbilical vein endothelial cells were exposed to 17β-estradiol and compared to control cultures with respect to levels of von Willebrand factor (vWF) released into the media after 3–5 days of incubation. The amount of functional vWF activity was assessed by ristocetin-induced platelet aggregation and by a radioreceptor platelet assay. vWF antigen was quantitated by immunoblotting. The DNA content of each monolayer was determined fluorometrically and used as a measure of cell number. By all assays, vWF levels in the media from the estradiol-treated endothelial cells were reproducibly and significantly higher when compared with control values. The amount of vWF produced by the cultured endothelial cells showed a dose–response effect to the estradiol added to the media. The estradiol-treated cells produced approximately 1.3 ± 0.30 μg vWF/ml/μg DNA at 2 ng estradiol/ml compared with control cultures that produced 0.75 ± 0.16 μg vWF/ml/μg DNA (p < 0.001). The estradiol-treated monolayers consistently contained slightly greater amounts of DNA than control cultures: 2.0 ± 0.10 μg versus 1.7 ± 0.12 μg DNA (p < 0.001). By multivariate analysis, however, the differences in cell number could only account for ≤10% of the elevation in the level of vWF that occurred in response to estradiol. By SDS-agarose electrophoresis and radioimmunoblotting, the vWF within the cytosol of the endothelial cells was found to possess a multimeric pattern similar to that found for either purified plasma vWF or vWF released into media overlaying endothelial cell cultures. Our studies indicate that estrogen directly stimulates endothelial cells to increase their rate of production of vWF and, in addition, causes a slight increase in endothelial cell replication. These data may bear on the observation that administration of estrogen to some women with von Willebrand’s disease causes an increase in their functional levels of vWF.

THE INTERACTIONS OF BLOOD with the vascular endothelium are important for maintaining a fluid state within the circulatory system and for initiating effective hemostasis. With respect to the latter phenomenon, the endothelium serves as a thromboresistant surface and synthesizes or reacts with a variety of plasma proteins that are important to the hemostatic process, namely: thrombin, protein C, thrombomodulin, plasminogen tissue activator, prostaglandin precursors and prostacyclin, collagen, fibronectin, tissue factor, mucopolysaccharides (including heparin), and α2-macroglobulin. The endothelial cell and the megakaryocyte synthesize von Willebrand factor (vWF)—an observation that has particular relevance to hemostasis because of the role of vWF in platelet adhesion and platelet aggregation at a site of vascular injury.

vWF circulates in plasma as a multimeric glycoprotein with molecular weights that range from approximately 1 to 10 x 10^6 daltons, depending on the method of analysis. Its direct involvement in the hemostatic process is supported by the observation that its absolute or functional absence causes the bleeding disorder, von Willebrand’s disease, which is characterized by mucocutaneous hemorrhage, a prolonged bleeding time, and usually a deficiency of antihemophilic factor (factor VIII; FVIII). The vWF released into media by cultured endothelial cells is in the form of functionally active oligomers that have a subunit structure identical to that of plasma vWF. More recently, evidence has been provided that the subunit is synthesized in precursor form as a 240,000–260,000 molecular weight species, which then undergoes proteolysis, with loss of about 20,000 daltons, before being joined by disulfide bonds to give the polymers found in media overlaying cultured endothelial cells.

A number of observations suggest that vWF in vivo, as well as in vitro, is intimately associated with the FVIII procoagulant moiety. Both functional activities of the FVIII/vWF complex are usually deficient in von Willebrand’s disease. Moreover, both functions copurify and appear tightly associated; however, when unusually high ionic strength solvents are used, especially 0.25 M CaCl₂, the two activities can be separated, suggesting the presence of two different molecular species that are associated by noncovalent bonds. In distinct contrast to the case for vWF, the site of synthesis of FVIII is unknown; attempts to demonstrate its functional presence in media from endothelial cell cultures have not been successful. Interestingly, however, a variety of perturbations appear to cause elevations of the functional levels of both FVIII and vWF in vivo, e.g., stress, epinephrine, and vasopressin or its analogues cause simultaneous increases of both functional activi-
Pregnancy, as well as the use of oral contraceptive medication, have also been frequently associated with elevated levels of FVIII/vWF. Indeed, because of these effects, von Willebrand’s disease may be ameliorated during pregnancy, especially in the third trimester.

Although a variety of agents have been tested, none have been found that consistently affect the synthesis and/or release of vWF by endothelial cells in vitro. A possible exception is the work of Wall et al., who showed that surface-adsorbed vWF appears to be released by endothelial cells in response to epinephrine. To date, the effect, if any, of estrogen on the production of vWF by endothelial cells has not been reported. The present work was undertaken to explore whether or not estrogens indeed cause an elevation of vWF in vitro, especially in light of recent findings in vivo.

Specifically, we examined the effect of estradiol on the production of vWF by cultured endothelial cells and, in addition, made preliminary observations about the structure and function of endothelial cytoplasmic vWF.

MATERIALS AND METHODS

Materials

Medium 199 with 25 mM HEPES, fetal bovine serum (FBS), and Hanks’ balanced salt solution (HBSS) were from GIBCO, Grand Island, NY. Collagenase (type CLS) and agarose-bound lactoperoxidase were from Worthington, Freehold, NJ. 17β-Estradiol, L-cysteine, calf thymus DNA, and bovine serum albumin (BSA) were from Sigma Chemical, St. Louis, MO. Cell culture plates and bottles were from Flow Laboratories, Hamden, CT. 3,5-Diaminobenzoic acid dihydrochloride was from Aldrich Chemicals, Milwaukee, WI. Carrier-free 125I was from Amersham Corp., Arlington Heights, IL. Ristocetin sulfate was obtained from H. Lundbeck and Co., Copenhagen, Denmark. Rabbit anti-human vWF was purchased from Calbiochem, La Jolla, CA.

Endothelial Cell Cultures

Endothelial cells were grown in culture using standard methods. Human umbilical veins were flushed with 50–100 ml of HBSS, then filled with 10 ml HBSS containing 0.1% collagenase and incubated for 20 min at approximately 37°C. Umbilical cords were lightly massaged, the collagenase solution collected, and the vein flushed with an additional volume of HBSS, which was drained and added to the first collection to bring the final volume to approximately 40 ml. The cell suspension was centrifuged at 280 g for 10 min, the supernatant discarded, and the endothelial cell pellet resuspended in 80% Medium 199–20% FBS; antibiotics were not used. The cells were then plated and grown at 37°C in a humid atmosphere of 95% air–5% CO2.

The effects of 17β-estradiol on endothelial cells were tested according to a standard protocol, except in those instances that are specifically noted. Endothelial cells from a single cord were plated in 12 2.4-cm wells of a multidish plate and allowed to grow to confluence in Medium 199–20% FBS. In some experiments, subconfluent cultures covering about 75% of the surface area were used. To initiate experiments, media was carefully aspirated and the monolayers were washed gently once with HBSS and overlayed with Medium 199 supplemented with 2% FBS and L-cysteine in a final concentration of 1.4 μg/ml. Half of the monolayers received supplements of either 17β-estradiol added to the media as a diluted solution in 6.7 × 10−6% (v/v) ethanol, or, in the case of control monolayers, the ethanol solution without estradiol. After 2 days, the media was removed, the monolayers washed carefully one time with HBSS, and 0.5 ml of media, with or without 17β-estradiol, was layered over the estradiol-treated and control monolayers, respectively. The cells were then incubated for an additional 3 days under the same physical conditions used for their growth.

Cytoplasmic vWF

Endothelial cytoplasmic vWF was obtained by in situ buffer wash of a 25 sq cm monolayer, extraction with 0.1% Triton X100 or 0.1% Nonidet P-40, and exhaustive dialysis against 0.01 M sodium phosphate–0.15 M NaCl buffer, pH 7.4. The vWF activity of the samples was tested in the ristocetin-cofactor assay,56 at the concentrations used, neither Triton X100 nor Nonidet P-40 interfered with the assay.

DNA Assay

At the end of the 3-day period of incubation, the medium was aspirated from the monolayers and centrifuged at 1,700 g for 20 min; 400 μl of media supernatant was routinely removed and frozen at −20°C for further assay. Each monolayer was lifted from its well after a 30–60-min incubation at 37°C in 0.5 ml of 0.05 M Tris-HCl–0.15 M NaCl–0.1% Na2EDTA, pH 7.4; during this incubation, the wells were also scraped gently with the tip of the pipette used for aspirating the cells. The DNA content of each such cell suspension was then measured using the method of Kissane,51 with slight modifications. In brief, the cell suspensions were centrifuged at 1,700 g for 15 min at 4°C, and 350 μl of supernatant were removed. Each pellet was sequentially extracted with 50 μl of ice-cold 0.6 N trichloroacetic acid; ice-cold 0.1 N potassium acetate solution in 95% ethanol; and 95% ethanol heated to 60°C for 15 min. Between each extraction, the cells were pelleted at 1,700 g for 15 min, and 50 μl of extract was removed. The pellets were evaporated to dryness in a 37°C oven for several days and then incubated at 60°C for 30 min in 0.1 ml of a 30 g/dl 3,5-diaminobenzoic acid dihydrochloride solution. Following this, 0.9 ml of 0.6 N perchloric acid was added, and the fluorescence of each sample was determined in a Perkin-Elmer Model 650-105 fluorescence spectrophotometer, using an excitation wavelength of 420 nm and an emission wavelength of 520 nm. Endothelial DNA values were determined by extrapolation from a standard curve of known concentrations of calf thymus DNA that had been treated exactly as above.

vWF Assay

The assay for vWF by a radioreceptor method has been previously described and was performed with minor modifications.3 Purified FVIII/vWF was iodinated with carrier-free sodium 125I using agarose-bound lactoperoxidase. One-tenth milliliter of each sample was placed in a 12 × 75 mm polystyrene tube to which was added sequentially 0.1 ml of 0.05 M Tris–0.15 M NaCl–0.1% BSA, pH 7.4; 0.1 ml 125I-labeled FVIII/vWF in Tris-NaCl-BSA buffer; 0.1 ml of Tris-NaCl-BSA buffer, containing ristocetin in a final concentration of 1 mg/ml; and 8 × 105 platelets in 0.1 ml of 0.05 M Tris–0.15 ml NaCl buffer, pH 7.4. After the platelet suspension was added, each incubation mixture was permitted to attain equilibrium for 2 hr at room temperature. Then, 1.5 ml of Tris-NaCl buffer, pH 7.4, chilled to 4°C, was added to each incubation tube and the platelets sedimented by centrifugation at 4,800 g and 4°C for 15 min. The supernatant was removed by aspiration, and the amount of radioactivity associated with each platelet pellet was determined using a Beckman Model 4000 gamma counter. FVIII/vWF concentrations...
were determined by extrapolation from a standard curve constructed from the determination of known levels of purified plasma FVIII/vWF in Medium 199-2% FBS and linearized using a log-logit transformation.

Levels of vWF in endothelial cell culture media were also measured in ristocetin-cofactor assays that were performed as previously reported. The vWF activity in each sample was determined by using 0.32 ml of the formalin-treated platelet suspension and 0.04 ml cell culture media. Agglutination was initiated with the addition of 0.04 ml of ristocetin (1.5 mg/ml final concentration) and the maximum rate of change of light transmission for each media sample was measured in a dual sample aggregometer (Sienco, Morrison, CO). Levels of ristocetin-cofactor were determined by extrapolation from a standard curve derived from assays of dilutions of purified plasma FVIII/vWF. Data from these assays were then linearized by double reciprocal transformations, as previously described.68

The levels of vWF in endothelial cell culture media were also measured immunologically according to previously described methods.5556 A standard reference curve for the assay was constructed using pooled plasma at 1/4, 1/6, 1/5, and 1/4 dilutions.

**Platelet Preparation**

Platelets used in either the radioreceptor or ristocetin-cofactor assays were obtained from the American Red Cross 2-4 days after collection from human volunteer blood donors and were formalin-fixed using a modification of the method of Head et al.57 Each platelet pack was washed 3 times by alternate centrifugation at 3000 x g and resuspension in at least 1,000 ml of wash fluid, consisting of 0.1 M Tris-HCl-0.14 M NaCl-0.005 M KCl-0.005 M dextrose-0.05% Na2 EDTA, pH 7.4. After incubation for 40-60 min at 37°C, a volume of 8% paraformaldehyde-0.28 M sodium phosphate, pH 7.4, equal to the volume of the initial platelet suspension, was added slowly and the suspension kept overnight at 4°C. The platelets were then washed 3 times with 0.05 M Tris-HCl-0.15 M NaCl, pH 7.4, made 4% in dimethyl sulfoxide, and stored frozen at -90°C in appropriate aliquots. For use, the frozen platelet suspension was thawed at 37°C, the platelets washed once in Tris-NaCl buffer to remove most of the dimethyl sulfoxide, and the platelet count adjusted using a hemacytometer under phase-contrast microscopy.

**Statistical Analysis**

Differences between the concentrations of DNA or vWF in media from control and 17β-estradiol-treated endothelial cell cultures were examined for statistical significance using Student’s t test.

**RESULTS**

All control monolayers from a given cord contained essentially identical amounts of DNA, suggesting that each well had approximately the same cell number; however, the mean cell number of monolayers from each umbilical vein differed from cord to cord. This variance was expected, as the cell suspensions were not adjusted to a standard concentration for plating and because the number of cells recovered, and their subsequent viability, varied considerably from cord to cord. Given these differences in plating and growth among cultures, the data in Table 1 show that the mean content of DNA in monolayers receiving 17β-estradiol was consistently greater when compared with matched control monolayers from the same cord. Pertinent to these measurements is that estradiol itself does not interfere with the fluorometric assay for DNA. For selected sets of endothelial cells, DNA content, as a reflection of cell number, was validated by showing that a correlation coefficient of 0.99 was obtained for DNA concentration (range 0.48-6.14 μg) versus endothelial cell counts (range 5.5 x 10⁶-74.3 x 10⁶) in 17 experiments. Moreover, our data in Table 1 show that the DNA measurements correlate well with the intentional plating of unequal volumes of cell suspension. By our assays of different cultures (n = 17), each endothelial cell contained about 8.5 ± 0.97 pg (mean ± 1 SD) of DNA, which is in excellent agreement with values reported for several different cell types,58 but slightly higher than the values for endothelial cells reported by Johnson and Erdos.59 In some of the cultures, cell replication was also quantitated by ³H-thymidine incorporation into trichloroacetic acid insoluble extracts of the endothelial cells. When compared to control values, these results, although less sensitive in our hands than the measurement of DNA, showed a 10% increase in the rate of DNA synthesis by the estradiol-treated cells (p < 0.01; total number of culture pairs, 56). Hence, based on the results from these two approaches, we concluded that estrogen caused a small increase in the replicative rate of endothelial cells.

Figure 1 shows good correlation between vWF concentrations measured by radioreceptor assay and by aggregation of fixed-washed platelets. vWF levels in

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<th>Table 1. Mean DNA Contents (± 1 SD) of Estradiol-Treated Monolayers Compared With Control Monolayers</th>
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*Seeded with three-fourths of the cell number contained in each corresponding control cell suspension.
†Corrected for cell number by multiplying 1.85 x 4/3.

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Fig. 1. Correlation of vWF concentrations in media determined by ristocetin-induced platelet aggregation with those determined by the platelet radioreceptor assay. Both methods were sufficiently sensitive for the detection of the small amounts of vWF released into the media by endothelial cells. The radioreceptor method of measurement estimates that normal human plasma contains a range of 8.3–24.9 μg vWF glycoprotein/ml and that 1.5 μg/ml is equivalent to approximately 0.1 U vWF activity/mL, defining the content of vWF activity in normal human plasma as 1 U/ml.

These observations, coupled with the fact that vWF concentration in media paralleled, whether expressed as percent binding versus log dilution or by log-logit transformation, the typical standard curve observed for purified human plasma vWF diluted in cell culture media and then assayed (Fig. 2). These observations, coupled with the fact that vWF could not be detected in media never exposed to cells, indicated that our assay was sufficiently sensitive and specific for detecting the relatively small changes of vWF concentration that might occur in response to estradiol.

Table 2 shows that at four concentrations of 17β-estradiol, the mean levels of vWF, without regard to DNA content or cell number, were consistently higher than the concentrations of vWF in media over corresponding control monolayers. Importantly, when cell number was taken into account, the mean vWF concentration at each of the four levels of estradiol was similarly elevated relative to vWF levels in control media. In Fig. 3, these data are expressed as the mean percent vWF increase when the 17β-estradiol-treated cultures were each compared to a corresponding control culture. We found the increases in vWF concentration in the media from the 17β-estradiol-treated cultures to be statistically significant at 500, 1,000, and 2,000 pg estradiol/ml (p < 0.001), but not at 250 pg/ml (p > 0.1). Figure 3 also demonstrates a dose–response effect in that the degree of increase in vWF is related to the concentration of 17β-estradiol used in each culture. A plateau in the increases of vWF concentration in response to 17β-estradiol occurred between 1,000 and 2,000 pg/ml and was not further affected by media containing 10,000 pg estradiol/ml. The latter estradiol dose gave a mean percent increase over control values that was essentially the same as that observed for the 2,000 pg/ml dose. Electroimmunoassay was used as another method for quantitating vWF in media from two sets of endothelial monolayers that had been treated with 2,000 pg estradiol/ml (each set was from a different cord and consisted of 6 cultures treated with estradiol and 6 controls). In both sets, the 17β-estradiol-treated cultures contained 44% more vWF than corresponding control media (2.4 ± 0.5 versus 1.6 ± 0.4% of normal; p < 0.01). As additional control experiments, media with and without estradiol were placed in wells that contained no endothelial cells. At no time during a 3–5-day incubation was vWF ever detected by the radioreceptor assay.
Fig. 3. Dose–response effect of 17β-estradiol on vWF production by endothelial cells. For each set of endothelial cell cultures, the percent increase of vWF concentration, when compared with a corresponding control, was normalized to the DNA content of that control culture. The lower curve shows the increase in vWF released into the culture media that could be attributed to the slight increase in cell number, as reflected by differences in DNA levels between the endothelial monolayers that received estrogen versus each corresponding control monolayer.

or by the agglutination of formaldehyde-treated platelets in the presence of ristocetin. Moreover, an immunoprecipitin reaction was never observed by electroimmunoassay.

As another test for the effect of estrogen, we deliberately distributed volumes of cell suspension unequally among wells to produce monolayers which, at the time of estradiol addition, would contain approximately three-fourths the cell number present in control layers. As shown in Table 2, despite the decreased cell number, which was also reflected by the DNA content for this set of monolayers, the mean concentration of vWF in media from the 17β-estradiol-treated cells was significantly higher than in controls, even without adjusting for the significantly lower DNA content of the estradiol-treated cells (2.5 ± 0.26 versus 1.9 ± 0.3 μg vWF/ml, p < 0.01). When the difference in DNA (1.4 ± 0.10 versus 0.49 ± 0.11 μg vWF/μg DNA, p < 0.001) was taken into account, the relative response to 17β-estradiol became even more apparent. This result virtually excludes the possibility that the elevated vWF levels in media from estradiol-treated cultures is due merely to a difference in endothelial cell number. Nevertheless, because results from DNA assays showed that 17β-estradiol-treated monolayers frequently contained more cells at the termination of experiments in which cells were plated equally in all wells, we considered that some fraction of the increased levels of vWF must be due to greater cell numbers as a consequence of increased replication. We therefore used a multiple regression technique to estimate the fraction of vWF increase that could be attributed to real differences in cell number. Figure 3 shows that, at every level of 17β-estradiol examined, the percent increase of vWF levels over control levels could be due only in part to a greater cell number. Importantly, at 2,000 pg of 17β-estradiol/ml, a difference in cell number could be responsible for no more than about 10% of the vWF response (at the 2,000 pg/ml estradiol level, n = 37, due to the exclusion of those monolayers that were deliberately plated with unequal numbers of cells). Based on these data, we concluded that the small increase in cell number in those cultures treated with 17β-estradiol accounted for only a small fraction of the increased level of vWF in the overlying media. Hence, the higher levels of vWF produced by endothelial cells in response to estrogen must occur mostly as a result of increased release or, more likely, increased synthesis.

After 3 days, media (3 ml) overlying 25 sq cm confluent endothelial monolayers contained approxi-
cytosol recovered from these monolayers contained 662 HARRISON AND McKEE 

assumed a confluent 25 sq cm endothelial cell monolayer and an

Figure 4 depicts the relative positions on the double-reciprocal 

S. aureus and a purified monospecific rabbit antibody
tg/ml. Figure 4 shows the electroimmunobLOTS of SDS- 

overlying media and endothelial cytoplasm contained 
evidence of high molecular weight vWF multimers. This result also suggests that the vWF multimers are 

assembled within human endothelial cells.25,26

DISCUSSION

Important to our findings here is the demonstration by others that endothelial cells do possess estrogen receptors.61 63 We now present evidence that 17β-
estraadiol directly induces the endothelial cell to augment its rate of production of vWF. In every instance, the addition of estradiol to endothelial cell cultures was associated with an increase of vWF protein in the media. At an estradiol concentration of 250 pg/ml, the difference between vWF concentrations in media from estradiol-treated and control endothelial cell cultures was not statistically significant; nevertheless, the dose-response effect observed between 500 and 2,000 pg/ml, where the differences observed were statistically important, suggests that the difference at 250 pg/ml was indeed real. Plasma estradiol concentrations of 250 pg/ml and greater, as measured by radioimmunoassay, are reported to occur at the midcycle surge in women of reproductive age.64 Levels of estradiol or estradiol-like steroids at least this high are also attained during pregnancy65 and in postmenopausal women using oral estrogens.66 Hence, the phenomenon that we observed in vitro might also occur in vivo.

The effects of the estrogenic hormones on plasma proteins have been noted.67 The association between pregnancy and the increase in levels of FVIII/vWF, in particular, have been reported by a number of observers,29,33,40,45 but in most of those studies, levels of vWF were not specifically determined, partly because reasonably objective assays of vWF were not available then. The amelioration of the hemostatic deficiencies in von Willebrand’s syndrome during pregnancy has received much attention, 29,33,40,44,45 but Adashi68 has cautioned that the phenomenon does not always occur

and has emphasized that women with von Willebrand’s syndrome must be followed carefully during pregnancy. Although the increased concentration of estrogens during pregnancy is thought to be responsible for the elevated levels of FVIII/vWF, only recently has it been reported that the administration of estrogen to women with von Willebrand’s disease will increase the plasma concentrations of FVIII/vWF-ristocetin cofactor activity, FVIII/vWF-related antigen, and FVIII procoagulant activity. FVIII:Ag, and FVIII:C as well as partially correct a prolonged bleeding time.46,47 Importantly, these changes correlate with a significant amelioration of the bleeding tendency. Our findings that estrogen directly stimulates the endothelium to produce vWF are in keeping with these observations and, in fact, may be the underlying explanation.

The relationship between the FVIII procoagulant activity and von Willebrand factor remains controversial, as FVIII has not been detected in media overlying endothelial cells.16,23,27,28 Our work, therefore, does not explain the observed increase in levels of FVIII in response to estrogens in vivo; however, an increase in FVIII may occur as a consequence of elevated levels of vWF, which some investigators have suggested serves as a carrier for FVIII,16,27 or may result from a direct stimulatory effect of estrogens on the cell that synthesizes FVIII. The observation that endothelial cell cytoplasm contains functionally active high molecular weight forms of vWF similar to that released into the media implies that assembly of subunits of this large glycoprotein can occur within the confines of the cell membrane. It may be that functionally active vWF species within the endothelium itself are at least as important as circulating vWF for promoting primary hemostasis. Forthcoming work will address the issue of whether the endothelial cytoplasmic content of vWF is also increased in response to estrogen.

It is now recognized that the vWF glycoprotein may play some role in atherogenesis, primarily based on observations that pigs homozygous for type I von Willebrand’s disease are resistant to atherosclerotic plaque formation.69 When this observation is considered with our findings here, and the recent report that men with documented coronary artery disease have significantly higher estrogen levels when compared with carefully matched controls,70 the relationship of estrogen to vWF production and its potential implications to the development of atherothrombotic disease should be considered. Although the estrogen levels in those men did not approach the concentrations used by us in vitro, the effects we report occurred after 3 days; it remains unknown what effects might occur during prolonged periods of sustained, low-level increases of estrogen.
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