Vitamin E attenuates biochemical and morphological features associated with development of chronic pancreatitis

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Submitted 4 August 2003; accepted in final form 1 March 2004

Gómez, José-Antonio, Xavier Molero, Eva Vaquero, Ana Alonso, Antonio Salas, and Juan-R. Malagelada. Vitamin E attenuates biochemical and morphological features associated with development of chronic pancreatitis. Am J Physiol Gastrointest Liver Physiol 287: G162–G169, 2004.—The objective was to investigate the effects of vitamin E on collagen deposition induced by Cyclosporin A (CsA) administration in rats with caerulein (Cr) pancreatitis. CsA transforms the fully regenerative, self-limited form of Cr pancreatitis into a chroniclike disease in conjunction with increased transforming growth factor (TGF-β) and myofibroblast proliferation. Vitamin E inhibits TGF-β release in mesangial cells and reduces CsA cytotoxicity. Wistar rats received CsA daily (20 mg/kg), and CR pancreatitis was induced on days 1 and 8 (Cr + CsA group). In a separate group, vitamin E (600 mg·kg−1·day−1) was administered starting 4 days before CsA. Three other groups received either vehicle, CsA, or Cr alone. Thioibarbituric acid-reactive substance (TBARS), 8-isoprostanes, and hyaluronic acid were measured in plasma obtained on the day the animals were killed (day 15). Pancreases were weighed and processed for light microscopy to assess connective tissue and myofibroblast number. Pancreatic homogenates were also assayed for collagen (hydroxyproline) and TBARS content. TBARS, 8-isoprostane, and TGF-β were elevated in Cr CsA rats but were effectively reduced by vitamin E. Vitamin E treatment greatly decreased these parameters. Vitamin E also decreased the fall in pancreatic weight observed in Cr + CsA pancreas. Pancreatic hydroxyproline and plasma hyaluronic acid were increased in Cr + CsA rats but were effectively reduced by vitamin E. Morphology showed improvement in fibrosis score and a decreased number of myofibroblasts in vitamin E-treated rats. Vitamin E reduces oxidative stress and collagen deposition during the development of experimental chronic pancreatitis. Adjuvant antioxidants may be of value in the treatment of chronic pancreatitis.

cyclosporin; caerulein; myofibroblasts; fibrogenesis

CSA nephrotoxicity is thought to be due to arteriolar vasoconstriction, tubule cell atrophy, and interstitial fibrosis (28, 50). CsA stimulates collagen synthesis in renal fibroblasts and promotes PDGF and TGF-β secretion by tubule cells, two cytokines involved in fibroblast activation (25). On the contrary, CsA induces antiproliferative and proapoptotic features in cells other than fibroblasts, such as tubulointerstitial, epithelial, and endothelial cells (14, 50).

The precise molecular mechanisms responsible for CsA-induced fibrogenesis are still undefined. They may involve impaired dephosphorylation of activated key proteins (27, 45), mitochondrial membrane permeability transition pore dysfunction (10), or enhancement of oxidative stress (29, 42, 56).

Vitamin E (α-tocopherol) is a lipophilic endogenous molecule with important antioxidant and nonantioxidant functions. Working in close connection with ubiquinone, vitamin C, and thiol redox cycles (such as glutathione and lipoic acid), vitamin E plays a leading role in controlling excess oxidative radical formation at cell membranes (9, 40), including mitochondrial membranes (8, 31, 59). Other physiological functions of vitamin E relevant to our study, which are partly or totally independent of its antioxidant capacity, include restoration of mitochondrial DNA degradation in the liver (34). On the basis of the above-mentioned evidence, we reasoned that vitamin E might prevent or mitigate the adverse effects of CsA on pancreatic regeneration and collagen deposition after Cr pancreatitis.

MATERIALS AND METHODS

Materials

Cr, hydroxyproline, malonaldehyde bis(dimethylacetal), vitamin E (RRR-α-tocopherol), and anti-α-smooth muscle actin monoclonal antibody were purchased from Sigma (St. Louis, MO). BCA reagent and bovine albumin standard for protein determination were from Pierce (Rockford, IL). Labeled streptavidin-biotin (LSAB) kit was

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from DAKO (Carpinteria, CA). CsA was from Sandoz Pharma (Barcelona, Spain). TGF-β1 test kit was from Genzyme (Cambridge, MA). 8-Isoprostane enzyme immunoassay (EIA) Kit and 8-Isoprostane affinity column were from Cayman Chemical (Ann Arbor, MI). Total lipids colorimetric test kit was from Spinreact (Sant Esteve de Bas, Spain).

Hyaluronic acid was measured with the Pharmacia HA Test radioimmunometric assay kit (Pharmacia and Upjohn Diagnostics, Uppsala, Sweden). All other chemicals were reagent grade and were obtained from common commercial suppliers.

Methods

Animals and experimental design. All experiments were performed in 300-g male Wistar rats housed in rack-mounted cages under conditions of constant temperature and a 12:12-h light-dark cycle. All procedures were approved by the Animal Experimentation Committee of the Institut de Recerca Vall d’Hebron.

A previously reported method (53) was used for induction of chroniclic pancreatic disease. Briefly, daily intraperitoneal doses of CsA (20 mg/kg diluted 1:4 in saline) were administered for 15 days, starting at day 0. On days 1 and 8, acute pancreatitis was induced by four intraperitoneal injections of Cr (20 μg/kg) at 1-h intervals (Cr + CsA group). Rats were euthanized at day 15, and pancreases were excised for histopathological examination (n = 10) or biochemical assays (n = 10). Three other groups underwent the same experimental procedure, except that they received either CsA, Cr, or vehicle (control group) alone. To examine the effects of vitamin E on pancreatic repair after acute pancreatitis, a separate group of rats followed the same protocol as the Cr + CsA group except that a high dose of vitamin E was administered daily by gavage (600 mg/kg), starting four days before the first pancreatitis induction until completion of the experiment (Cr + CsA + VitE group). To compensate for the stress associated with animal manipulation, all other groups received an equivalent volume of saline by gavage on a daily basis. Dietary vitamin E has been shown to increase vitamin E tissue concentration in a dose-dependent manner (19). Better results are often obtained when high doses are administered (6, 21, 51), whereas adverse side effects only occur with extremely high doses for long periods of time (1). Before rats were euthanized, blood was withdrawn by cardiac puncture using heparinized needles and syringes and placed in siliconized tubes, and plasma was obtained after low-speed centrifugation of total blood. Special care was taken in the process of blood manipulation, from extraction to plasma separation, to minimize the risk of cell lysis. Samples obtained to measure α-tocopherol, thiobarbituric acid-reactive substance (TBARS), or 8-isoprostanes were protected from oxidation by adding butylated hydroxytoluene (0.01%) during the extraction. Plasma samples were freed from platelets by means of an additional centrifugation (5,800 g for 10 min at 4°C) to avoid platelet interference with the TGF-β1 assay.

To verify that vitamin E treatment did not reduce acute pancreatitis severity, Cr + CsA rats and Cr + CsA + VitE rats (n = 5/group) were euthanized 6 h after acute pancreatitis induction. Amylasemia, tissue edema, and myeloperoxidase activity were measured.

Pancreatic edema was assessed by measuring pancreatic water content as the percentage of total wet weight. A sample of the head of the pancreas was wet weighed, desiccated at 160°C for 24 h, and reweighed.

Biochemical determinations. Amylase activity was assessed by the enzymatic colorimetric determination of pancreatic α-amylase (Senti
cine, Milan, Italy) using an automated analyzer (Olympus AU5400; Olympus Optical, Tokyo, Japan). Aliquots (200 μl) of 1.50 dilution of the sample were used in the assay.

Tissue myeloperoxidase activity was measured in pancreatic homogenates using a previously-described method (54).

Plasma α-α-tocopherol was determined by HPLC. Briefly, 200 μl of plasma were placed in acid-washed test tubes. After deproteinization with ethanol (400 μl), 2 ml hexane were added and the mixture was vortexed for 60 s and then centrifuged for 20 min at 4°C. Supernatants were dried under nitrogen, resuspended in 200 μl methy

anol, and subjected to reverse-phase HPLC equipped with a 440 diode array detector (Kontron Instruments, Zurich, Switzerland). Detection of α-tocopherol was accomplished with ultraviolet detection at 284 nm. Calibration of the HPLC system was performed using fresh solutions of α-tocopherol in ethanol.

For hydroxyproline assay, total pancreases were excised, trimmed of fat and lymph nodes, weighed, frozen, and lyophilized. A powder was made in a mortar from the dried residue and resuspended in ice-cold distilled water (100 mg/ml). Pancreatic homogenates were prepared by serial use of a motor-driven shearer (Tissue Tearer 985–370, Biospec Products, Drewel, WI) and Dounce tissue homog

eizers on ice.

Total protein and hydroxyproline content were quantified from crude homogenates. Protein concentration was determined by biconi

chonic acid reagent (Pierce).

Hydroxyproline content was determined as described (50). Briefly, 200 μl of crude homogenate were placed in an ampoule containing 2 ml 6 N HCl and sealed. Hydrolysis was performed at 110°C for 16 h. The hydrolysate was dried, and the sediment was redissolved in 2 ml distilled water and dried again. This latter step was repeated three times before the residue was resuspended in 3 ml 0.2 M citrate buffer (pH 2.2). One milliliter of the sample or of hydroxyproline standards was placed in a pyrex test tube and mixed with 1 ml chloramine T solution prepared as described by Rojkind and Gonzalez (45). After 20 min, the reaction was stopped by the sequential addition of 0.5 ml sodium thiosulphate (2 M), 1 ml NaOH (1 N), and 2 g NaCl. Proline was extracted in 2.5 ml toluene by shaking the contents for 30 s. The aqueous fraction was placed in a boiling water bath for 30 min which was then cooled to room temperature. Hydroxyproline was extracted in 2.5 ml toluene and then determined by the use of Ehrlich’s reagent.

To measure biologically active TGF-β1 concentration in platelet-free plasma, samples and standards were acidified using HCl for 1 h and then neutralized to pH 7.0–7.4 immediately before sample loading for enzyme-linked immunosorbent assay (Genzyme, Cambridge, MA) (30).

The soluble nonprotein component of the extracellular matrix (ECM) hyaluronic acid has been found to be a useful marker of fibrosis (16, 33, 39, 48). Hyaluronic acid was measured in plasma as a collagen-independent marker of increased ECM turnover by means of a radiometric assay (Pharmacia HA test).

Plasma lipid peroxidation was assessed by measuring both TBARS (52) and 8-epi PGF-α (8-isoprostanes), an eicosanoid of nonenzymatic origin produced by direct oxidation of phospholipids by reactive oxygen species (ROS) (32).

Thiobarbituric-reactive substances were measured in platelet-free plasma following published procedures (47, 60). Briefly, 100 μl of samples were added to a reaction mixture containing 700 μl H2O, 200 μl SDS 8.1%, 1,500 μl of 20% acetic acid (pH 3.5), and 1,500 μl of 0.8% thiobarbituric acid. Samples were then heated to 95°C for 1 h and centrifuged at 3,000 g for 15 min. Absorbance of the clear supernatants was determined spectrophotometrically at 532 nm. Standard curves were prepared using malonaldehyde bis(dimethylacetal).

TBARS were also determined in pancreas. Briefly, fresh pancreatic tissue was minced and homogenized (100 mg/ml) in ice-cold 1.15% KCl containing 0.01% butylated hydroxytoluene and stored at −80°C until assayed. Homogenates (100 μl) were assayed in duplicate for TBARS content. From the initial homogenate, 200 μl were dessicated to dryness by heating at 160°C for 16 h and then weighed. Results are provided as nanomoles per 100 mg dry wt.

Total plasma 8-epi PGF-α was determined using a commercially available EIA kit (Cayman Chemical) according to the manufacturer’s specifications. Samples were purified with the help of affinity columns (Cayman) before the EIA assay.
Morphology

Pancreatic specimens were fixed in 10% formalin and embedded in paraffin. Several sections were cut and stained with hematoxylin and eosin (H & E). Connective tissue accumulation was assessed using a semiquantitative morphological scoring system adapted from Demolis et al. (12) that grades 0 for normal pancreas and 3 for extensive interstitial fibrosis. Histological gradings were performed on 10 successive fields within each section at a magnification of $\times100$. The mean of the scores of all fields was recorded for each specimen. Acinar atrophy was also scored accordingly (12), taking 0 for normal pancreas and 3 for extensive acinar atrophy. When indicated, immunohistochemistry was performed with the monoclonal anti-$\alpha$-smooth muscle actin (αSMA) antibody to identify myofibroblasts, i.e., intrastitial nonvascular αSMA-positive cells (SMAc) with fibroblastic shape, using the avidin-biotin-peroxidase complex system (LSAB kit; DAKO). Quantitative analysis of interstitial SMAc was performed at $\times400$ final magnification. Ten nonoverlapping fields were evaluated for each animal in each treatment group.

All histological slides were examined by the participating pathologist (A. Salas), who was unaware of the tissue source.

Statistical Analysis

Values are expressed as means ± SE. The unpaired Student’s $t$-test was used when two variables were compared. When more than two variables were present, group means were compared using ANOVA followed by Fisher’s protected least-significant differences test. If variances were not found to meet equality criteria, variables were compared in groups of two using Student’s $t$-test. Differences were regarded as significant when $P < 0.05$.

RESULTS

Acute Pancreatitis

At 6 h postinduction, vitamin E treatment did not appear to affect the severity of acute pancreatitis in Cr + CsA rats, as assessed by pancreatic water content (78.2 ± 1 vs. 76.5 ± 1% water content; $P = 0.4$), amylasemia (21 ± 2 vs. 18 ± 2 U/ml; $P = 0.3$), or pancreatic myeloperoxidase activity (290 ± 20 vs. 270 ± 22 mU/mg protein; $P = 0.3$).

Plasma Vitamin E

CsA treatment in rats with pancreatitis induced an increment in total plasma lipids at the end of the observation period (503 ± 27 mg/dl) compared with control rats (306 ± 37; $P < 0.05$). Vitamin E treatment in Cr + CsA rats normalized plasma lipid concentration (272 ± 37). No effect on plasma lipids was observed either after CsA treatment alone (261 ± 10) or after Cr pancreatitis (247 ± 10). According to these findings, plasma concentrations of vitamin E, TBARS, and 8-isoprostaness were adjusted to total lipid concentration. Two weeks after the first pancreatitis induction, vitamin E concentration was reduced in Cr pancreatitis with or without CsA treatment (Fig. 1). Vitamin E supplementation resulted in a large increment in plasma vitamin E concentration at the end of the experimental period.

Effects of Vitamin E on Plasma Lipoperoxidation

Two weeks of CsA treatment to rats, with or without Cr pancreatitis, resulted in increased lipoperoxidation in blood, as demonstrated by the high levels of TBARS in plasma (Fig. 2). Vitamin E treatment in Cr + CsA rats fully prevented the increment in plasma TBARS, indicating an efficacious antioxidant effect of vitamin E counteracting CsA-enhanced oxidative stress. Measurement of 8-isoprostane is now accepted as a reliable method for the assessment of oxidative stress in vivo (32). Results on lipid peroxidation were fully confirmed by measuring 8-isoprostane concentration (Fig. 2). It is noteworthy that CsA treatment alone induced large increments in these two parameters indicative of lipid peroxidation.

Effects of Vitamin E on Lipoperoxidation in Pancreatic Tissue Samples

Lipoperoxidation in pancreatic tissue, as measured by the TBARS assay, paralleled the findings observed in plasma, except that the impact of CsA treatment alone on pancreatic TBARS concentration was not as marked (Fig. 3). Treatment with CsA to rats with Cr pancreatitis resulted in a large increment in pancreatic TBARS concentration that was largely prevented by vitamin E supplementation. In addition, pancreas from rats with Cr pancreatitis showed a moderate increase in TBARS concentration compared with control rats, probably reflecting residual ongoing inflammation and tissue remodeling 7 days after the last pancreatitis induction.

Effects of Vitamin E on the Profibrogenic Activity of CsA

As we previously reported (53), plasma TGF-β was persistently elevated in rats with Cr pancreatitis receiving CsA. In addition to its antioxidant effect, vitamin E treatment was also associated with a reduction in plasma TGF-β (Fig. 4). In addition to its effects on plasma lipoperoxidation, CsA treatment to rats without pancreatitis was also associated with increased plasma TGF-β concentration.

Collagen pancreatic content, as measured by hydroxyproline concentration in pancreas, was increased in Cr + CsA rats compared with control or Cr rats (Fig. 5). Vitamin E significantly reduced the increment in collagen deposition induced by...
Vitamin E treatment also reduced hyaluronic acid concentration in blood (Fig. 5). Thus vitamin E effectively reduced two biochemical parameters indicative of increased ECM turnover: hydroxyproline, measured as an index of pancreatic collagen content (the representative fibrillar ECM protein), and hyaluronic acid, a soluble nonprotein element of the ECM. However, at this time point (2 wk after the first pancreatitis), vitamin E was unable to fully prevent CsA-induced fibrogenesis, because hydroxyproline and hyaluronic acid were still elevated in Cr + CsA + VitE rats compared with the Cr group.

Pancreatic Morphology

Total pancreatic weight is a simple but reliable method for estimating the degree of pancreatic regeneration after injury. Vitamin E administration greatly ameliorated the pancreatic weight loss induced by CsA in Cr pancreatitis rats. However, in agreement with hydroxyproline and hyaluronic acid findings, pancreatic weight of vitamin E-treated rats was still far below the weight observed in control or Cr rats (Fig. 6).

Vitamin E clearly enhanced pancreatic regeneration in Cr + CsA + VitE rats, as demonstrated by light microscopy inspection (Fig. 7). The degree of acinar atrophy and the amount of pancreatic connective tissue were greatly reduced by vitamin E administration. As previously reported (53), CsA administration alone did not produce significant effects on pancreatic structure, except for minor modifications such as cytoplasm vacuolization in some acinar cells.

Blind semiquantitative assessment confirmed these observations. Connective tissue accumulation was decreased by vitamin E treatment compared with Cr + CsA rats (1.2 ± 0.1 vs. 2.2 ± 0.2; P < 0.05, n = 10), and the degree of acinar atrophy was also improved (1.8 ± 0.2 vs. 2.5 ± 0.2; P < 0.05, n = 10). Immunostaining for SMAc was used to detect and quantify activated myofibroblasts. Compared with Cr + CsA rats, vitamin E treatment was associated with a reduction in the number of activated myofibroblasts present in the pancreas.

CsA in Cr pancreatitis rats. Moreover, vitamin E treatment also reduced hyaluronic acid concentration in blood (Fig. 5). Thus vitamin E effectively reduced two biochemical parameters indicative of increased ECM turnover: hydroxyproline, measured as an index of pancreatic collagen content (the representative fibrillar ECM protein), and hyaluronic acid, a soluble nonprotein element of the ECM. However, at this time point (2 wk after the first pancreatitis), vitamin E was unable to fully prevent CsA-induced fibrogenesis, because hydroxyproline and hyaluronic acid were still elevated in Cr + CsA + VitE rats compared with the Cr group.

Pancreatic Morphology

Total pancreatic weight is a simple but reliable method for estimating the degree of pancreatic regeneration after injury. Vitamin E administration greatly ameliorated the pancreatic weight loss induced by CsA in Cr pancreatitis rats. However, in agreement with hydroxyproline and hyaluronic acid find-
settings and experimental animal models reinforce this argument (35, 36, 54).

ROS are normally produced in cells in a number of biological processes where they regulate critical physiological and pathophysiological functions (18). Elements engaged in a complex molecular machinery that prevent ROS from becoming harmful are termed endogenous antioxidants. Oxidative stress is the molecular and cellular damage resulting from excessive ROS production or from reduced endogenous antioxidants.

Assessment of oxidative stress in vivo usually relies on the determination of stable oxidatively modified molecules (17). However, these molecules may be generated in too low amounts or may be metabolized too quickly to permit reliable measurements. In our experimental model, the presence of increased plasma and pancreatic TBARS as well as circulating 8-isoprostanes several days after the last pancreatitis induction clearly indicates that CsA induces persistent lipoperoxidation. Vitamin E reduces both TBARS and 8-isoprostanes. In addition, we have shown that 8-isoprostanes (bioactive no enzymatic generated prostaglandin-like compounds derived from ROS-catalyzed peroxidation of arachidonic acid) (32) are produced in response to CsA treatment. In rats with pancreatitis, an excess of CsA-induced oxidized bioactive lipids such as 8-isoprostanes may contribute to generating further damage to the pancreas.

Administration of CsA to patients has been shown to increase plasma lipids (5, 20). Interestingly, in our experimental model, CsA treatment in rats with Cr pancreatitis was also associated with a marked rise in plasma lipids. On the contrary, total plasma lipid concentration in normal rats was not modified by CsA treatment, despite its effects on plasma lipoperoxidation. Furthermore, vitamin E supplementation to Cr + CsA rats reverted the increment in plasma lipids induced by CsA.

We have also shown that CsA treatment in Cr pancreatitis induces persistent increments in TGF-β bioactivity, which are again reversed by vitamin E treatment. TGF-β involvement in fibrogenesis is widely documented (55). In Cr pancreatitis, TGF-β infusion enhances matrix accumulation (52), whereas...
Antibodies raised against TGF-β tend to diminish it (37). Previous studies (25) reported CsA-induced release of TGF-β in the kidney and in supernatants of cultured tubule cells. On the other hand, vitamin E abrogates TGF-β secretion induced by angiotensin II or thromboxane in glomerular mesangial cells (49). In the liver, vitamin E supplementation inhibits TGF-β gene expression (41). In an in vivo model of pulmonary fibrosis, recent observations that provide further support to our work indicate that vitamin E supplementation decreases TGF-β gene expression and pulmonary hydroxyproline content (7). Oxidative stress may either be the cause or the result of TGF-β activity (11, 49). Both TGF-β and oxidative stress are independently able to activate pancreatic stellate cells (3, 4, 38), manifested by “de novo” αSMA expression and by phenotypic transdifferentiation into fibroblast-like cells termed myofibroblasts. These same signals, oxidative stress and TGF-β, induce apoptosis in epithelial cells that results in parenchymal atrophy (15). Thus the histological features induced by CsA treatment in our model (increased ECM, infiltrating fibroblasts, and parenchymal atrophy) tantalizingly resemble the characteristic picture of chronic pancreatitis.

Vitamin E was also shown to reduce CsA-induced lipoperoxidation, TGF-β bioactivity, and the number of activated myofibroblasts. These effects run parallel to a reduction in ECM deposition and acinar cell atrophy. Vitamin E, the most abundant lipid-soluble antioxidant, is present in relevant amounts in biological membranes, being positioned as a first-line defense element against oxidative attack by ROS. α-Tocopherol concentration rises as the daily amount of vitamin E in the diet increases (19, 31). In addition, the concentration of vitamin E in mitochondrial membranes is inversely related to the production of oxidative radicals (31). It is of particular interest to note that one of the targets of CsA is, precisely, the mitochondrial membrane, a major source of ROS in non-phagocytic cells and where vitamin E displays its full antioxidant function. Not surprisingly, quite a number of studies has
reported on the benefits of vitamin E supplementation on CsA-induced cytotoxicity (2, 42, 56).

Vitamin E reduced the number of myofibroblasts in the repairing pancreas, an effect also observed in other fibrogenic conditions (6). Pancreatic myofibroblast activity is thought to be the cause of increased ECM in chronic pancreatitis. However, myofibroblasts participate in normal repair processes aimed at fully restoring pancreas architecture after injury. CsA may exert its action on a number of cell types (endothelial, vascular smooth muscle, epithelial, and acinar cells) that might subsequently affect myofibroblast activity. In addition, direct effects of CsA on pancreatic myofibroblasts should also be considered. One possibility is that CsA-induced persistent increments in ROS, TGF-β, or both may keep myofibroblasts in their activated profibrogenic state. Moreover, CsA inhibition of mitochondrial-induced apoptosis may prevent activated myofibroblasts from being removed from the healing tissue. Excess of matrix synthesis by redundant spared myofibroblasts might impair the necessary tissue remodeling that precedes full restoration (22). By counteracting CsA-induced oxidative stress and TGF-β bioactivity, vitamin E may reverse this trend and facilitate myofibroblast apoptosis. The pathways regulated by vitamin E that are able to modulate pancreatic fibrogenesis merit further investigations.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Pilar Chacón and María Isabel Guilera for excellent technical work in the determination of vitamin E concentrations.

GRANT

This work was supported in part by Grant FIS 01/1401 and Generalitat de Catalunya 2001SGR 00389.

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