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Inflammation Research

Hydrogen peroxide induces apoptosis of chondrocytes; involvement of calcium ion and extracellular signal-regulated protein kinase

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Abstract. *Objective:* Recent observations demonstrated that reactive oxygen species facilitate cartilage degradation. We demonstrated that hydrogen peroxide (H_2O_2) caused inhibition of proteoglycan synthesis, induction of apoptosis and stimulation of extracellular signal-regulated protein kinase (ERK) of the chondrocytes (Inflamm Res 48: 399–403, 1999). To determine whether activation of ERK is involved in the induction of chondrocyte apoptosis, we examined the signal transduction pathways in this hydrogen peroxide induced apoptosis.
Design: Bovine articular chondrocytes were cultured. To determine the induction of apoptosis, Annexin V staining and terminal deoxynucleotidyl transferase were used. The activity of caspase-3 was measured using an apopain assay kit. Intracellular Ca^{2+} imaging was observed after fura2-AM loading.
Results: Hydrogen peroxide enhanced annexin V positive apoptotic cells and caspase-3 activity, which is an executor of apoptosis. Hydrogen peroxide also enhanced intracellular Ca^{2+} and preincubation with the intracellular Ca^{2+} chelator protected chondrocytes against hydrogen peroxide-induced cell apoptosis, indicating that an increase in the cytosolic Ca^{2+} plays a decisive role in this action. When ERK activity was blocked with geldanamycin and PD098059, increased apoptosis was evident.
Conclusion: Hydrogen peroxide induces chondrocyte apoptosis via Ca^{2+} signaling, and ERK is involved in these signal transduction pathways.

Key words: Annexin V – Apoptosis – Chondrocytes – MAP kinase

Introduction

Chondrocytes, differentiated cells embedded in an avascular matrix, are exposed to partial pressures of oxygen and exhibit a predominantly anaerobic metabolism. Accordingly, chondrocytes are susceptible to the attack of reactive oxygen species (ROS). We have investigated the induction of ROS, such as superoxide anion (O_2^-), nitric oxide (NO) and peroxynitrite (ONOO-), in bovine articular chondrocytes and found that these ROS play an important role in cartilage degradation [1–4]. In addition to these ROS, Schalkwijk, et al. reported the significant role of hydrogen peroxide (H_2O_2) in cartilage destruction in vivo [5]. Recently, we demonstrated that hydrogen peroxide induced the apoptosis of the chondrocytes [6].

The two most widely described forms of cell death are necrosis and apoptosis. The criteria for determining whether a cell is undergoing apoptosis versus necrosis include distinct morphological changes, as well as alterations in biochemical and molecular markers, such as terminal deoxynucleotidyl transferase (TdT, TUNEL) and annexin V [7]. Another distinction between necrotic and apoptotic cells is the requirement for new mRNA and protein expression during the early stages of apoptosis, which is restricted to specific cell types and stimuli. Of those, caspase proteases are essential elements of apoptotic signaling pathways [8]. In the present study, we examined whether hydrogen peroxide promotes caspase 3 activity, which degradates a variety of cytoplasmic and nuclear proteins [9], in the chondrocytes.

The production of ROS has been thought for a long time to adversely affect the physiology and survival of a cell. There is now a growing body of evidence to suggest that ROS can influence the growth, as well as death, of animal cells in vitro through the activation of multiple signalling pathways that influence the cytotoxicity observed in affected cells [10, 11], including the phosphorylation cascades leading to the activation of mitogen-activated protein kinases (MAPKs). MAPKs, which include extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38 subfamilies, are important regulatory proteins through which various extracellular signals are transduced into intracellular events [12].

Treatment of chondrocytes with hydrogen peroxide resulted in the induction of apoptosis accompanied by transient activation of ERK [6]. However, the important question

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remained, whether transient activation of ERK is involved in the induction of chondrocyte apoptosis.

The purpose of this study is to determine the signal transduction pathways that participate in this hydrogen peroxide induced apoptosis.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin/amphotericin B and neomycin were obtained from Gibco (Grand Island, NY). Fetal bovine serum (FBS) was purchased from HyClone (Logan, UT) and hydrogen peroxide was purchased from Sigma (St. Louis, MO). Bis-(o-aminophenoxy) –N,N,N,N′– tetraacetic acid acetoxymethyl ester (BAPTA-AM) was purchased from Wako Pure Chemical Industries. Annexin V-FITC and fura-2 (fura-2-AM) were purchased from Molecular Probes (Eugene, OR) and Treigen, Inc.(Gaithersburg, MD), respectively.

PD098059 and geldanamycin were purchased from Calbiochem (La Jolla, CA) and dissolved in DMSO and stored as 10 mM stock solutions.

Chondrocyte culture

Articular cartilage slices were taken from the condylar ridge of metacarpophalangeal joints of freshly slaughtered calves approximately 10 months of age. Care was taken to exclude the underlying marrow. Chondrocytes were obtained by enzymatic dissociation [13]. They were seeded at a density of 2×10^5 cells/ml for the measurement of PG synthesis and ERK kinase in 96-well plates (Becton Dickinson, NJ). The culture medium was DMEM, supplemented with penicillin-streptomycin-fungizone and 10% FBS at 37 °C in a 5% CO_2 environment. Six or twelve replicates per treatment group were run for each experiment.

Apoptosis assays

To determine the induction of apoptosis, chondrocytes were seeded at a density of 2×10^3 cells/ml in 24-well culture plates. Culture conditions were described above. Annexin V staining was accomplished following the product instructions (Clontech, Palo Alto. CA). In brief, 10 µl of Annexin V-FITC (1 mg/ml) and 10 µl of propidium iodide (PI, 2.5 mg/ml) were added to the samples for 5 to 15 min in the dark. The cells were analyzed under a laser scanning confocal microscope (MRC 2400 LSX imaging system; Bio-Rad, Hercules, CA) equipped with an argon laser adjusted to an output of 250 mW at 488 nm excitation, 525 nm emission. A minimum of 100 cells were counted. We defined Annexin V positive and PI negative cells (Annexin+/PI–) as apoptosis.

We also determined the chondrocyte apoptosis using TUNEL assay, as described previously [6].

Measurement of caspase activities

The activity of caspase-3 was measured using the FluorAce™ apopain assay kit (Bio-Rad, CA). Briefly, cells were collected by centrifugation, washed twice with phosphate-buffered saline without Ca^{2+} or Mg^{2+}, and lysed in apopain lysis buffer (10 mM Hepes buffer, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM dithiothreitol, 1 mM PMSF, 10 µg/pepstatin A, 10 µg/aprotinin, 20 µg/leupeptin). After centrifugation at 10,000 × g for 10 min at 4 °C, the supernatants were removed. 40 µl of reaction buffer, 10 µl of 7-Amino-4-trifluoromethyl coumarin (AFC)-conjugated peptide substrate (acetyl-Asp-Glu-Val-Asp-AFC, Ac-DEVD-AFC, [14]) and 950 µl of water were added on the cell lysates. After incubation at 30 °C for 1 h, reactions were stopped by adding 2 ml of ice-cold PBS, and the levels of released AFC were measured using a spectrofluorometer (CytoFlour series4000, Perseptive Biosystems Framingham, MA) with excitation at 400 nm and emission at 505 nm. To quantify the enzyme activity, we prepared a calibration curve by plotting AFC concentration (nanomoles) vs fluorescence change according to the manufacturer's instructions.

Ca^{2+} Imaging

Bovine chondrocytes were plated onto Flexiperm-Discs (W.C. Haraeus) in MEM, supplemented with 10% FCS and cultured for 5 days. Videomicroscopy and Ca^{2+} measurements were then carried out at room temperature and loaded with 1 µM of Fura2-AM as previously described [15]. The incubation medium contained (in mmol/l) NaCl 140, KCl 5, $MgCl_2$ 1.2, $CaCl_2$ 1, glucose 10, and HEPES-NaOH buffer 20, pH 7.4. The digital fluorescence-imaging system used was an Argus-50 (Hamamatsu Photonics) that allowed 7 cells to be monitored simultaneously. Video frames were digitized, integrated in real time, and finally processed off-line to convert fluorescence data in Ca^{2+} maps. These maps were generated using the 340/380 nm excitation wavelength ratio method. The mean values in each area of interest were calculated from sequential images. Quantitative temporal analyses in spatially restricted areas were thus obtained throughout the experiment, and fluorescent intensities were measured. To calibrate the ratio (R = 340/380) into the concentration of Ca^{2+}, we used calcium calibration buffer kits (Molecular Probes, OR).

Statistical analysis

Results are presented as the mean ± SD. Significant differences were determined by Student's t-test. A significance level of $P < 0.05$ was used to reject the null hypothesis.

Results

Hydrogen peroxide-treated cells exhibited morphological alterations consistent with apoptosis including shrinkage and membrane blebbing, as we previously reported [6]. These hallmarks of apoptosis were not evident in the control (untreated) cells.

One of the earliest markers of apoptosis is the translocation of phosphatidylserine from the inner to the outer layer of the plasma membrane [16]. This change can be detected by testing for binding of the protein annexin V to the cell surface [7]. To determine whether cells undergo this early apoptotic change, chondrocytes were cultured in the presence of hydrogen peroxide. We found that hydrogen peroxide (300 µM) induced annexin V binding over the course of a 6 hr incubation (Fig. 1). In addition to this time course study, we examined the dose-dependency in hydrogen peroxide-induced apoptosis after 12 h of treatment. Although little cytotoxicity was evident at concentrations below 100 µM, there was a dose-dependent increase in annexin V positive cells up to 500 µM of hydrogen peroxide (Fig. 2).

We also examined the activation of caspase-3 using Ac-DEVD-AFC as a substrate and found that the treatment of hydrogen peroxide (300 µM) for 3 h caused the activation of caspase 3 (Table 1).

In the presence of 300 µM of hydrogen peroxide, cytosolic Ca^{2+} significantly increased in chondrocytes. This response was preceded by a short period of latency (5–10 s), and was characterized by a steep onset to the maximal Ca^{2+} level, followed by a slower decline to the pre-existing values (Fig. 3). Fluorescent analyses showed that for 30 determina-

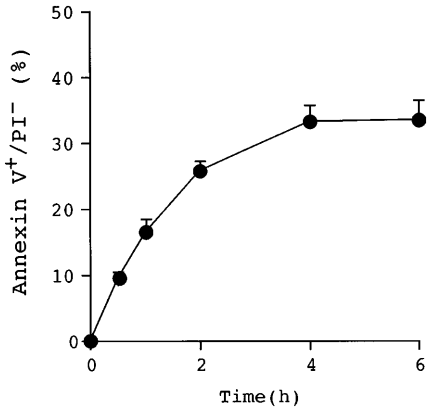

Fig. 1. Effect of hydrogen peroxide on apoptosis of chondrocytes. Bovine articular chondrocytes were treated with hydrogen peroxide (300 μM) for 6 h. At the indicating time, the vital cultures were incubated with annexin V-FITC and PI. The cells were subsequently analyzed using the BioRad MRC 2400 confocal scanning laser microscope. We defined Annexin V positive and PI negative cells (Annexin+/PI–) as apoptosis. Representative results of 3 different experiments are shown. The circles and bars show the mean ± SD of 12 determinations.

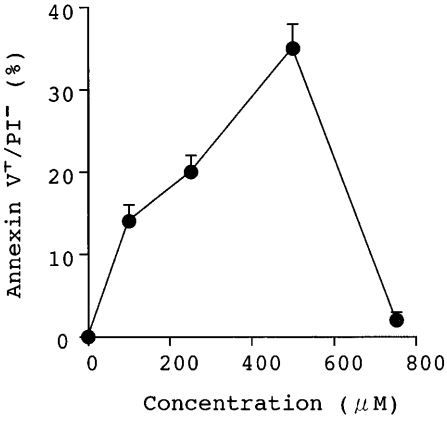

Fig. 2. Effect of hydrogen peroxide on apoptosis of chondrocytes. Chondrocytes were cultured in the presence of a different concentration of hydrogen peroxide for 12 h. Apoptosis was determined by the annexin V binding assay and the percent of apoptotic cells was determined. Representative results of 2 different experiments are shown. The circles and bars show the mean ± SD of 12 determinations.

tions, the emission ratio (R) peaked at a level of 0.92 ± 0.12 from a baseline value of 0.55 ± 0.14. When cells were preincubated for 30 min with BAPTA/AM, which is a membrane-permeable, non-chelating tetra (acetoxymethyl) ester. Following uptake by the cell, BAPTA/AM is hydrolyzed by cytosolic esterases, yielding the active chelator BAPTA, which remains intracellular [17]. BAPTA/AM abolished hydrogen peroxide-increased cytosolic Ca^{2+} (Fig. 4). We also examined the effect of BAPTA/AM on hydrogen peroxide-induced apoptosis and found that apoptosis was inhibited with BAPTA/AM (Fig. 5).

We next investigated the influence of ERK activation on hydrogen peroxide-induced apoptosis in chondrocytes employing the inhibitor of ERK. Geldanamycin, which is an ansamycin antibiotic and disrupts tyrosine kinase signaling

Table 1. Effect of hydrogen peroxide on caspase-3 activity

	Exp.1	Exp. 2
Control	82 ± 1	95 ± 1
Hydrogen peroxide	455 ± 2*	529 ± 2*

Chondrocytes were cultured in the absence (control) or presence of hydrogen peroxide (300 mM) for 3 h, and enzyme activity (nM/10^6 cell) was measured using the FluorAec™ apopain assay kit with Ac-DEVD-AFC as a substrate. Data are expressed as the mean ± SD of 4 wells. Two different experiments were curried out with a different preparation of bovine chondrcytes. * $p > 0.01$ vs control.

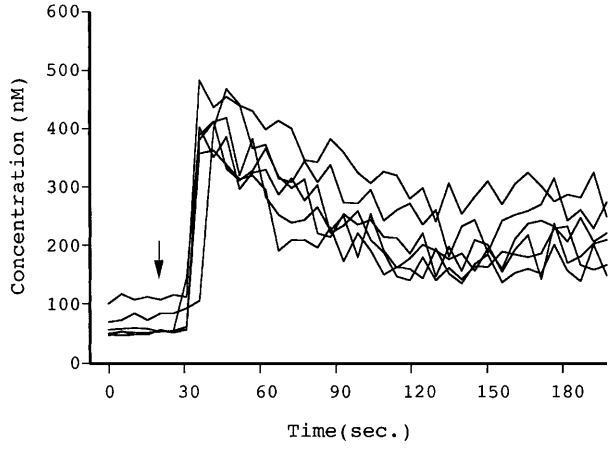

Fig. 3. Hydrogen peroxide-mediated Ca^{2+} mobilization in articular chondrocytes. The cells were loaded with fura-2 AM, and hydrogen peroxide was added (arrowhead). The ratio signals (340/380 nm) were recorded, and the concentration was calibrated, using calcium calibration buffer kits. One representative experiment of four is shown.

[18], treatment resulted in an increase in the numbers of annexin V positive cells. Some degree of annexin V positive cells were evident as early as 1 h after treatment, and a significant percentage of annexin V positive cells were observed by 4 h. When cells were pretreated with 20 μM of PD098059 for 1 h before the addition of 300 μM of hydrogen peroxide, there was a significant increase in annexin V positive cells (Fig. 6). Neither geldanamycin (2 μM) nor PD098059 (20 μM) alter the percentage of apoptotic cells (data not shown). To further confirm the induction of apoptosis, we performed a TUNEL assay to detect DNA breakage that occurs during stage of apoptosis. Geldanamycin leads to an increase in the numbers of TUNEL-positive cells (Fig. 7).

Discussion

Growing evidence suggests that chondrocyte apoptosis plays an important role in the degradation of articular cartilage in cases of human osteoarthritis [19] and rheumatoid arthritis [20]. We demonstrated that hydrogen peroxide induced apoptosis of chondrocytes using the "TUNEL" assay [6]. In the present study, we demonstrated the time- and dose-dependent induction of apoptosis with hydrogen peroxide using an annexin V binding assay. It should be noted that the

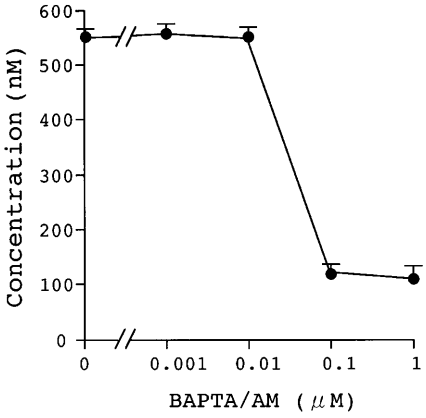

Fig. 4. Effect of BAPTA/AM on hydrogen peroxide-increased cytosolic Ca^{2+}. When cells were preincubated for 30 min with different concentrations of BAPTA/AM, BAPTA/AM abolished 300 μM of hydrogen peroxide-increased cytosolic Ca^{2+}. Representative results of 2 different experiments are shown. The columns and bars show the mean ± SD of 12 determinations. *P < 0.01.

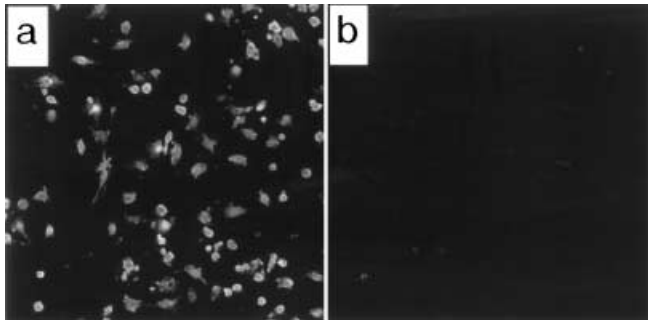

Fig. 5. Effect of BAPTA/AM on hydrogen peroxide-mediated Ca^{2+} mobilization in articular chondrocytes. Cells were pre-cultured in the presence of BAPTA/AM (1 μM) for 24 h. Hydrogen peroxide (300 μM) was added for 6 h, and apoptosis was determined by the annexin V binding assay. After 1 h, the vital cultures were incubated with annexin V-FITC and PI. The cells were subsequently analyzed using the BioRad MRC 2400 confocal scanning laser microscope. a: Merged image of annexin V fluorescence and PI. b: Pretreatment with BAPTA/AM. ×400.

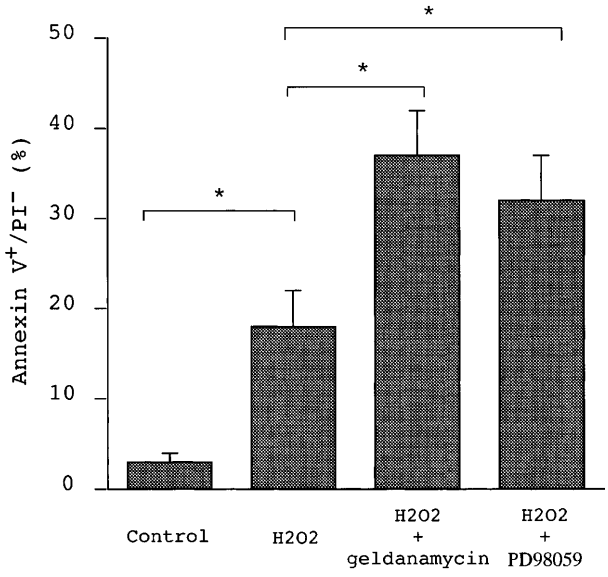

Fig. 6. Effect of geldanamycin and PD098059 on hydrogen peroxide-induced apoptosis. Chondrocytes were precultured in the presence of geldanamycin (2 μM) or PD098059 (20 μM) for 1 h before the addition of 300 μM of hydrogen peroxide. Cells were further cultured for 1 h, and they were incubated with annexin V-FITC and PI. The percent of apoptotic cells was determined. Representative results of 2 different experiments are shown. The columns and bars show the mean ± SD of 12 determinations. *P < 0.01.

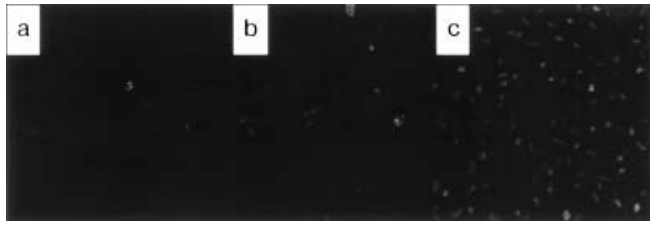

Fig. 7. Effect of geldanamycin on hydrogen peroxide-induced apoptosis. Chondrocytes were precultured in the presence of geldanamycin (1 μM) for 6 h before the addition of 100 μM of hydrogen peroxide. Cells were further cultured for 12 h and apoptosis was determined by a "TUNEL" assay. a: non-treated control, b: hydrogen peroxide without geldanamycin pretreatment. c: hydrogen peroxide with geldanamycin pretreatment.

ratio of annexin V positive cells reduced with over 500 μM of hydrogen peroxide. A higher concentration of hydrogen peroxide could cause necrosis of the chondrocytes rather than apoptosis, and hydrogen peroxide-induced apoptosis may occur only under a very narrow concentration range.

We also demonstrated the hydrogen peroxide-enhanced caspase-3 activity, which is a executor of apoptosis [6], using caspase-3/apopain kit. Caspase 3/apopain is derived from proenzyme CPP32 at the onset of apoptosis and plays a pivotal role in programmed cell death. Among the many members of the interleukin-1 β-converting enzyme protease family recently described throughout the literature, apopain exhibits the highest similarity to the C. elegans cell death gene in both sequence homology and substrate specificity [21]. Therefore, apopain activity appears to be a suitable indicator of critical apoptosis biochemistry. Previous researchers have reported that hydrogen peroxide inhibits caspase activity in Jurkat cells [22] and concluded that hydrogen peroxide might act by inhibiting the caspases directly. Our data do not support such a conclusion.

The significance of hydrogen peroxide-induced apoptosis is unclear. Although it is possible that hydrogen peroxide inhibits proteoglycan synthesis by causing chondrocyte apoptosis, there is currently insufficient evidence to conclude that the effect of hydrogen peroxide on cartilage degradation can be accounted for solely by an increase in apoptosis. It is more likely that hydrogen peroxide inhibits metabolic pathways that are important for chondrocyte signal transduction. Recent reports show the involvement of mitochondrial permeability and the release of cytochrome C in hydrogen peroxide-induced apoptosis in other cell lines [23, 24].

We next examined the intracellular Ca^{2+} because disruption of cellular Ca^{2+} homoeostasis and the release of Ca^{2+}

from intracellular storage into the cytosol is evident in hydrogen peroxide-induced cell death in other systems [25]. This is the first report, to our knowledge, showing enhanced cytosolic Ca^{2+} with hydrogen peroxide in the chondrocytes. Preincubation with the intracellular Ca^{2+} chelator (BAPTA/AM) strongly protected chondrocytes against hydrogen peroxide-induced cell apoptosis. Therefore, an increase in the cytosolic Ca^{2+} concentration obviously plays a decisive role in the cytotoxicity of hydrogen peroxide towards articular chondrocytes.

Previous studies from our laboratory suggested that the activation of ERK was an important factor in determining the survival of chondrocytes after treatment with hydrogen peroxide [6]. In the present study, through the use of an inhibitor of ERK, we have addressed the relative contributions of this kinase in influencing the survival of hydrogen peroxide-treated chondrocytes. Tyrosine kinase inhibitors geldanamycin inhibits the activation of ERK, i.e. the Ras/Raf-1/Mek pathway [18]. This hydrogen peroxide-induced apoptosis was markedly enhanced with the treatment of geldanamycin. ERK2 activation was inhibited by PD098059 [26, 27], suggesting that early ERK activation acts as a factor for surviving oxidant injury against the cartilage. Aikawa et al. have recently provided evidence that ERK activation protects cardiac myocytes from apoptotic death after oxidative stress [28]. However, it is not clear in this study whether enhanced hydrogen peroxide-induced apoptosis with these inhibitors is really mediated via ERK or independent of ERK activity. Further study is necessary to determine the precise role of ERK in hydrogen peroxide-induced apoptosis.

In conclusion, 1) hydrogen peroxide induces chondrocyte apoptosis via Ca^{2+} signalling. 2) ERK is involved in these signal transduction pathways.

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