

A Thrombelastograph whole blood assay for clinical monitoring of NSAID-insensitive transcellular platelet activation by arachidonic acid

ROGER C. CARROLL, ROBERT M. CRAFT, JACK J. CHAVEZ, CAROLYN C. SNIDER,
STUART J. BRESEE, and ELI COHEN

KNOXVILLE, TENNESSEE and NILES, ILLINOIS

Optical platelet aggregation (OPA) with platelet-rich plasma (PRP) was compared with a Thrombelastograph (TEG) whole blood assay for monitoring arachidonic acid (AA)-induced platelet activation. Assays were performed on 47 interventional cardiology and 24 general surgery patients receiving aspirin therapy for cardiovascular disease, as well as 48 volunteers asked to take nonsteroidal anti-inflammatory drugs (NSAIDs) or 12 volunteers on chronic NSAID therapy unrelated to diagnosed cardiovascular disease. Whole blood TEG monitoring of NSAID inhibition detected NSAID-insensitive AA activation of platelets in a significantly higher number of cardiology (23%) and surgery (25%) patients and normal volunteers on chronic NSAID (25%) therapy relative to normal subjects not on chronic NSAID therapy (0%). Whole blood NSAID insensitivity was observed with cyclooxygenase-I inhibitors, such as aspirin and ibuprofen; was not affected by Celebrex, a cyclooxygenase-II inhibitor; but was completely inhibited by thromboxane-receptor antagonists. This was not due to platelet NSAID insensitivity, because complete inhibition of AA-activation responses in PRP was observed with either TEG or OPA assays. We confirmed that thromboxane B₂ formation in PRP from NSAID-insensitive subjects was completely inhibited by NSAIDs. However, significant amounts were formed in whole blood from NSAID-insensitive subjects, but not in whole blood from NSAID-sensitive subjects. Thromboxane formation after AA addition was not found in washed blood cells with 90% reduced platelet counts or in leukocyte-rich buffy coat fractions, but could be restored by addition of PRP. NSAID-insensitive activation was inhibited by nordihydroguaiaretic acid, with an IC₅₀ of 30 μmol, implicating 12- and/or 15-lipoxygenases in this transcellular pathway. (*J Lab Clin Med* 2005;146: 30–35)

Abbreviations: AA = arachidonic acid; ADP = adenosine diphosphate; KH = kaolin and heparinase; MA = maximum amplitude; NSAID = nonsteroidal anti-inflammatory drug; NDGA = nordihydroguaiaretic acid; OPA = optical platelet aggregation; PRP = platelet-rich plasma; PPP = platelet-poor plasma; SD = standard deviation; TEG = Thrombelastograph®; TxB₂ = thromboxane B₂

From the Departments of Anesthesiology and Medicine, University of Tennessee Graduate School of Medicine, Knoxville, Tennessee; and Haemoscope Corporation, Niles, Illinois.

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Reprint requests: Roger C. Carroll, PhD, University of Tennessee Graduate School of Medicine, 1924 Alcoa Highway, Knoxville, TN 37920; e-mail: RCarroll@utk.edu.

Several platelet-independent, transcellular pathways for nonsteroidal anti-inflammatory drug (NSAID)-insensitive platelet activation by arachidonic acid (AA) have been suggested.^{1,2} In 1 study, 40% of patients undergoing coronary artery bypass did not have prolonged bleeding times in response to aspirin therapy.³ This was associated with 12-hydroxyicosatetraenoic acid

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synthesis and increased platelet adhesion, possibly through a transcellular aspirin-insensitive 12- and/or 15-lipoxygenase pathway.⁴ Aspirin-resistant conversion of AA into platelet-activating thromboxane has also been linked to a monocyte prostaglandin H synthase insensitive to cyclooxygenase inhibitors and inducible by inflammatory stimuli.^{5,6} Transcellular activation by platelet-leukocyte cross-talk in whole blood with physiological calcium has also been suggested.⁷

We have recently tested the Thrombelastograph (TEG) heparin-anticoagulated whole blood assay (TEG Haemostasis Analyzer 5000; Haemoscope, Niles, Ill), which measures platelet interaction with a fibrin network analogous to platelet aggregation under physiological calcium conditions.⁸ This TEG assay correlates with optical platelet aggregation (OPA). Similar to OPA, the TEG assay is sensitive to both clopidogrel adenosine diphosphate (ADP) inhibition and NSAID inhibition of AA-mediated platelet activation.

In this study, we correlated and compared the TEG whole blood assays to OPA for the detection of NSAID inhibition in 2 different patient populations, as well as in normal volunteers. In addition, we report on the use of TEG to distinguish NSAID-insensitive from NSAID-sensitive transcellular platelet activation in whole blood.

METHODS

Materials. AA and ADP were obtained from Chrono-Log Corporation (Havertown, Penn) and made up and stored as concentrated stock solutions in accordance with the manufacturer's instructions. Specific thromboxane A₂ receptor (TxA₂-R) antagonists, SQ29548 and ICI 192605, were obtained from BIOMOL Research Laboratories (Plymouth Meeting, Penn). Nordihydroguaiaretic acid (NDGA) was obtained from Oxford Biomedical Research (Oxford, Miss). Stock 1000-fold concentrated solutions were composed in ethanol.

Human subjects. Our Institutional Review Board reviewed the protocol used to obtain informed consent from 47 candidates for interventional cardiology and 24 elective general surgery patients on aspirin therapy for cardiovascular disease. Patients were excluded from the study if they had thrombocytopenia, diagnosed hemophilia or chronic bleeding problems (eg, peptic ulcer), or decreased liver function. For studies on the affect of NSAIDs unrelated to diagnosed cardiovascular disease, 60 normal volunteers were recruited, 12 already taking a continuous regimen of NSAIDs at various dosages and types and 48 taking a 325-mg uncoated aspirin dose and/or standard doses of other cyclooxygenase I or II inhibitors for a period equivalent to peak pharmacokinetic effect (1–2 hours with uncoated aspirin or ibuprofen, 3–5 hours with Celebrex) before giving a blood sample.

Thrombelastograph assays. Blood was drawn into Vacutainer tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ), anticoagulated with 14.7 U/mL heparin, and

assayed within 2 hours for platelet functional responses to 1 mmol AA. The platelet response, which is analogous to OPA-measured aggregation, was used as an assay for NSAID inhibition as described previously.⁸ The TEG assay with heparin-anticoagulated blood was used to determine the maximum amplitude (MA) of clot shear elasticity. The MA is proportionate to the extent of platelet activation elicited by 1 mmol AA and represents the interaction of the activated platelet glycoprotein IIb/IIIa complex with a fibrin network formed by a reptilase–Factor XIIIa mixture (provided by Haemoscope). Responses were compared with the maximal MA of the same blood clotted by thrombin with the addition of heparinase and kaolin (MA_{KH}), in accordance with the manufacturer's instructions. A percent MA response (TEG %MA) was calculated by subtracting the MA without platelet activator (MA₀) from the MA with AA (MA_{AA}), dividing by MA_{KH} minus MA₀, and then multiplying the result by 100%. This %MA value has been shown to correlate well with percent aggregation as determined by OPA.⁸

Optical platelet aggregometry. Heparin-anticoagulated blood was also used to isolate platelet-rich plasma (PRP) and platelet-poor plasma (PPP) by differential centrifugation for assay of OPA using a Chrono-Log Aggregometer with AggroLink software (Chrono-Log). OPA assays of maximum aggregation for 10 minutes after the addition of 1 mmol AA and calculation of percent aggregation were carried out as described previously.⁹ OPA was assayed without platelet count adjustment.

Cell fractionation. Further cell fractionation was carried out after removing the PRP layer, as described earlier. For some experiments, the leukocyte-rich buffy coat was also collected separately. The blood cell fractions were diluted 1:1 with a balanced salt solution (Normosol; Abbott Laboratories, North Chicago, Ill) containing 6 U/mL heparin. The samples were centrifuged at 100 × g for 20 minutes at room temperature, and the top layer containing platelets was discarded. This procedure was repeated twice more, until platelet counts were reduced to <10% of the original whole blood sample. The top of the red blood cell fraction minus the buffy coat was also removed, to further reduce white blood cell count to <15% of the original whole blood counts. Blood cell counts in various fractions were determined on an Ichor/Plateletworks analyzer (Helena Laboratories, Beaumont, Tex).

Thromboxane B₂ (TxB₂) assays. TEG samples with reaction conditions as described in Results were run in quadruplicate. After MA values were obtained, the samples were collected into Microfuge tubes (Beckman Coulter, Fullerton, Calif). The samples were centrifuged at 10,000 × g for 5 minutes at room temperature, and the sera was collected and frozen at –70°C. TxB₂ concentrations in serum samples were assayed with an enzyme immunoassay kit (Oxford Biomedical Research) following the manufacturer's directions.

Statistics. Paired *t*-tests were used to establish significant changes from individuals' baseline platelet function. The significance of group differences was tested using unpaired *t*-tests for parametric data, Mann-Whitney *U*-tests and Kruskal-Wallis tests for nonparametric data, and χ^2 tests for categorical data. Statistical tests were done with StatView 4.0 software (SAS Institute, Cary, NC).

Table I. Occurrence of NSAID insensitivity in different patient populations and normal volunteers

	Cardiology (n = 47)		Surgery (n = 24)		Normal (n = 48)		Normal, chronic NSAID (n = 12)	
	Yes	No	Yes	No	Yes	No	Yes	No
NSAID insensitive	11	36	6	18	0	48	3	9
Percent occurrence	23		25		0		25	

χ^2 testing of the data shows significantly ($P < .0001$) less occurrence of NSAID insensitivity in normal subjects not on chronic NSAID relative to other groups.

RESULTS

As reported in a previous study,⁸ there is generally agreement between OPA and TEG with almost complete NSAID inhibition of normal subjects' 1-mmol AA TEG response ($> 50\%$ MA off NSAIDs, reduced to $< 12\%$ MA after NSAID treatment). However, despite profound NSAID inhibition of the mean OPA response to AA, as expected,¹⁰ with all of the patients' PRP samples in the current study (OPA = $2.5\% \pm 2.5\%$ [standard deviation]), we found that 24% overall (Table I) gave 1-mmol AA TEG responses, with whole blood not showing significant inhibition (mean TEG %MA = $79\% \pm 19\%$). We define this positive TEG response associated with a negative OPA response to AA as a type of whole blood NSAID insensitivity. There were no significant differences in smoking status ($P = .39$), diabetes mellitus ($P = .12$), use of NSAIDs besides aspirin ($P = .64$), overall gender ($P = .32$), or in overall age (mean, 68 vs 65 years; $P = .39$; unpaired *t*-test) for NSAID-sensitive subjects compared with NSAID-insensitive subjects.

In normal volunteers, we found this OPA and TEG assay disagreement in 3 out of 60 subjects. All 3 were on chronic NSAID therapy for joint pain. Chronic NSAID users composed 12 of our 60 normal subjects unrelated to a diagnosis of cardiovascular disease. The 3 normal NSAID-insensitive subjects were all over age 55 years and included 1 female. This would indicate a similar 25% insensitivity in subjects on chronic NSAID therapy as in our patient populations. The TEG assay indicated this NSAID insensitivity only in whole blood, because PRP isolated from the same sample and used in the TEG assay showed complete inhibition of AA activation, in agreement with OPA (Fig 1).

Data in Table II, obtained with the whole blood TEG assay with 3 NSAID-insensitive normal volunteers as well as 6 NSAID-insensitive patients, shows that this activation is also not inhibited by concurrent celecoxib therapy to inhibit both cyclooxygenase I and II,^{11,12} but is completely inhibited by 2 different TxA_2 -receptor antagonists added in vitro. This NSAID insensitivity was not altered by having the normal volunteers switch from ibuprofen to aspirin or by quadrupling the aspirin dose from 325 to

1300 mg. These data indicate that the NSAID-insensitive AA metabolite was likely to be TxA_2 . This was confirmed by measurement of TxB_2 , the stable metabolite of TxA_2 . NSAID-sensitive subjects ($n = 6$) formed an average of 310 ± 25 ng/mL TxB_2 after activation by AA in the TEG whole blood assay, which was reduced to control baseline levels of 0–2 ng/mL after subjects took an NSAID. In contrast, NSAID-insensitive subjects' whole blood in the TEG assay formed an average of 117 ± 25 ng/mL TxB_2 despite taking NSAIDs when AA activated, compared with 0–2 ng/mL baseline. This TxB_2 formation was not blocked by the TxB_2 antagonists but was inhibited by the lipoxygenase inhibitor NDGA. At 100- μmol concentrations, NDGA almost completely inhibited the TEG %MA response and TxB_2 formation in these whole blood AA-activated TEG samples.

This NSAID-insensitive TEG %MA AA, but not ADP, activation is inhibited by NDGA with an IC_{50} close to 30 μmol , as shown in Figure 2. The reported IC_{50} of NDGA for 12- and 15-lipoxygenases is 30 μmol , compared with 100 μmol for cyclooxygenase,^{13,14} but we saw very little inhibition of the AA response of whole blood even at 200 μmol in terms of both TEG %MA and TxB_2 formed (257 ± 28 μmol vs 220 ± 35 μmol ; $n = 3$) in the absence of NSAIDs.

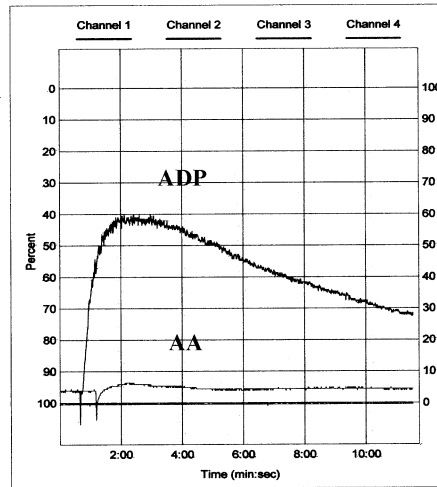
Table III shows that no significant TxB_2 formation was observed in PRP fraction from 3 NSAID-insensitive subjects after AA addition. However, TxB_2 formation was restored in PRP samples reconstituted with either washed blood cells or with the buffy coat fraction, but not with blood cells minus the buffy coat fraction. Very little TxB_2 formation after AA addition was observed in the washed cells or buffy coat mixed with PPP and having $< 10\%$ of the original platelet count. TxB_2 formation in whole blood was also observed in the absence of clot formation induced by reptilase and Factor XIIIa.

DISCUSSION

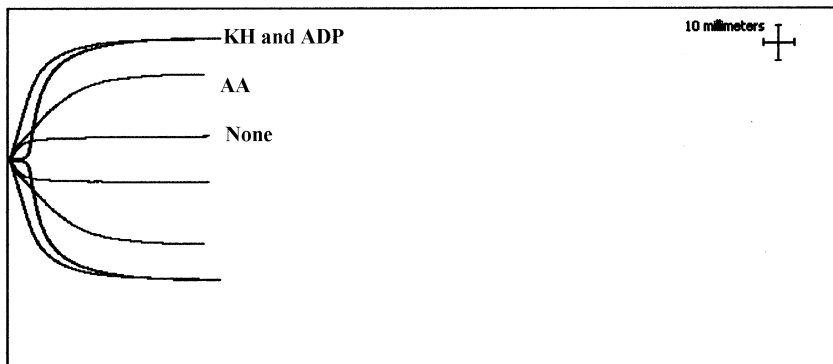
NSAID inhibition of 1 mM AA, yielding $> 50\%$ aggregation in the absence of NSAID inhibition, is profound, and $< 5\%$ aggregation responses are seen after aspirin ingestion.¹⁰ This agrees with our previously reported data for OPA with a mean of $66\% \pm 12\%$ aggregation off

A. PRP-OPA

Name	Normal, NSAID			
ID	Date 03/31/2004			
Hosp.				
Channel	1	2	3	4
Instrument	Opt	Lum	Opt	Lum
Reagent	1 μ M ADP		1 mM AA	
Stirrer	1000	1000	1000	1000
Gain				
Amplitude	55%	0%	2%	0%
Slope	134	1	36	1
Comments				



B. Whole Blood-TEG



C. PRP-TEG

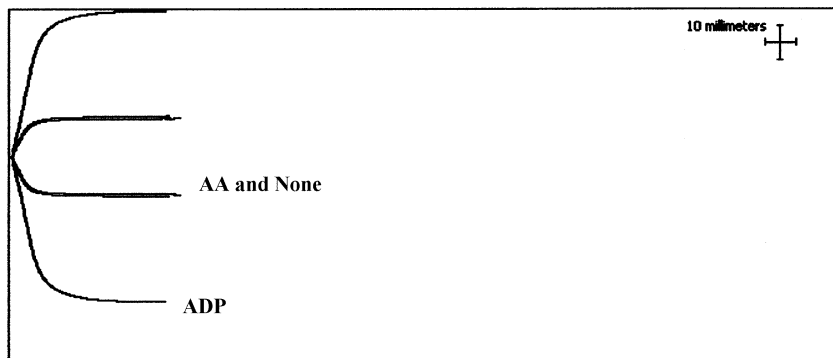


Fig 1. OPA and TEG responses of an NSAID-insensitive normal donor blood sample to 1 μ mol ADP and 1 mmol AA. (a) OPA responses as indicated by the traces; %MA for each activator is indicated as amplitude in the data analysis left of the traces. (b) TEG MA responses in whole blood to ADP and AA compared with MA₀ and MA_{KH} clotted samples giving the maximum possible MA_{KH}. (c) TEG MA responses using same PRP as in (a). In (b) and (c), the vertical bar in the right upper corner represents 10 mm MA, and the horizontal bar represents 5 minutes. Blood was drawn within 2–4 hours of administration of a 500-mg dose of ibuprofen.

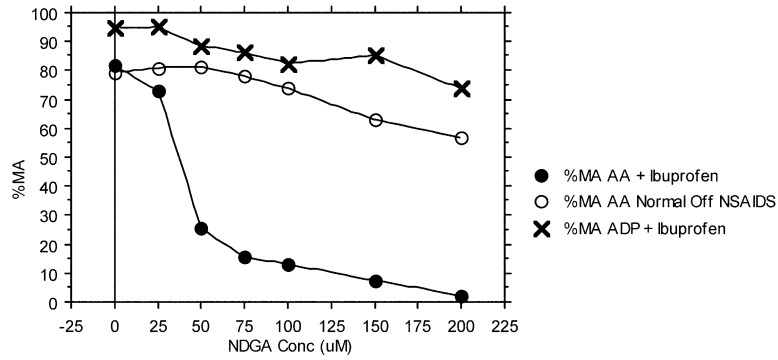


Fig 2. An NSAID-insensitive normal subject on ibuprofen versus a normal NSAID-sensitive subject off NSAIDs: 12- and 15-lipoxygenase inhibitor (NDGA) titration of AA and ADP responses. Whole blood samples from an NSAID-insensitive subject (filled symbols) and an NSAID-sensitive subject off NSAIDs (open circles) were assayed by TEG for either AA (circles) or ADP (X's) responses in terms of %MA. Samples were preincubated for 10 minutes at room temperature with the indicated concentration of NDGA added from 1000-fold concentrated stock ethanol solutions. Vehicle controls show no effect of this small concentration of ethanol. Thus experiment is representative of 3 different normal subjects, each assayed 3 times.

Table II. Effect of a cyclooxygenase II inhibitor, txA_2 receptor antagonists, or a lipoxygenase inhibitor, on NSAID-insensitive TEG %MA and TxB_2 formation after AA addition

Inhibitor	TEG %MA (mean \pm SD)	TxB_2 (ng/mL) (mean \pm SD)
None	67 \pm 15	117 \pm 25
Celecoxib (600-mg oral dose)	62 \pm 19	112 \pm 32
1 μmol ICI 192,605	5 \pm 4	103 \pm 21
1 μmol SQ 29548	3 \pm 2	107 \pm 14
100 μmol NDGA	6 \pm 6	5 \pm 4

Assays were done with normal subjects ($n = 3$) on ibuprofen or aspirin therapy and patient subjects ($n = 6$) already on low-dose aspirin and celecoxib therapy. Subjects were assayed before and after taking celecoxib as a 600-mg oral dose with blood sampling 3-5 hours later. Other inhibitors were added ex vivo to blood samples. Thromboxane receptor antagonists were added and samples assayed without further incubation. NDGA was added, and the samples were incubated for 10 minutes at room temperature before assays.

NSAIDs versus a $2\% \pm 2\%$ aggregation response on NSAIDs.⁸ However, in this study we noted a 24% incidence of NSAID insensitivity in our patient groups by the TEG whole blood assay using AA but not by OPA done with PRP. This finding was not seen in normal volunteers not on chronic NSAID therapy but had a similar incidence in the normal subjects on chronic NSAID therapy unrelated to diagnosed cardiovascular disease. This suggests that NSAID insensitivity is a consequence of chronic NSAID therapy in these patient groups. A high rate of NSAID insensitivity developed in patients with thrombotic stroke on chronic aspirin therapy monitored over time by the combined results of ADP, epinephrine, collagen, and AA-mediated OPA.¹⁵ In this earlier study,

Table III. Thromboxane formation in NSAID-insensitive fractionated whole blood after 1 mmol AA addition

Blood cell fraction	TEG %MA	TxB_2 (ng/mL)
Unfractionated blood	67 \pm 18	97 \pm 12
Unfractionated blood (no reptilase + Factor XIIIa)	0	113 \pm 15
PRP	2 \pm 2	1 \pm 1
Washed cells + plasma (<10% platelet count)	0	2 \pm 2
Washed cells + PRP	55 \pm 15	65 \pm 18
Washed cells minus buffy coat + PRP	3 \pm 1	2 \pm 1
Buffy coat + PPP (3-6 WBCs $\times 10^3/\mu\text{L}$)	0	1 \pm 1
Buffy coat + PRP	54 \pm 16	58 \pm 11

Normal NSAID-insensitive subjects ($n = 3$) were assayed on 4 separate occasions. Blood was fractionated and assayed as described in Methods. Data given are means standard deviations (SD).

NSAID insensitivity was indicated by a normal aggregation response to any of the aforementioned platelet activators. It was not reported whether any of the NSAID-insensitive subjects exhibited AA-mediated aggregation responses, which is the more direct assay for NSAID inhibition.¹⁰ In a more recent study, NSAID insensitivity in patients with cardiovascular disease on 325 mg/day aspirin therapy was defined by the combined criteria of $\geq 70\%$ ADP-mediated and $\geq 20\%$ AA-mediated OPA responses.¹⁶ This was compared with a whole blood assay using a platelet function analyzer (PFA-100; Dade Behring Inc., Deerfield, Ill) and a collagen and/or epinephrine assay cartridge. The investigators found that 18 of 325

patients were aspirin-insensitive by OPA and that 31 of 325 were aspirin-insensitive by PFA-100 whole blood assay, with only 4 patients insensitive by both tests. The Ultegra Rapid Platelet Function Assay-ASA (Accumetrics, San Diego, Calif), another whole blood platelet function test, was used to determine the aspirin responsiveness of 151 patients treated with aspirin and clopidogrel before nonurgent percutaneous cardiology intervention.¹⁷ Aspirin insensitivity was detected in 19% of the patients and was associated with greater postprocedure myonecrosis. Aspirin-insensitive formation of urine 11-dehydrothromboxane B₂ has been also been linked in the Heart Outcomes Prevention Evaluation (HOPE) study to a higher risk for cardiovascular ischemic events during a 5-year follow-up.¹⁸

The NSAID insensitivity seen in 24% of the whole blood samples in our study does not reflect direct platelet insensitivity, because both OPA and TEG assays with PRP are completely inhibited. Our results indicate transcellular platelet activation by formation of TxA₂ mediated by the transcellular interaction of white blood cells and platelets. This transcellular activation seems to be mediated by 12- and/or 15-lipoxygenases, because it is sensitive to NDGA with an IC₅₀ of 30 μmol. Identification of the specific cell types, enzyme variants, and lipoxygenase AA intermediates involved in this transcellular activation is the subject of current studies. We are also comparing other whole blood assays of platelet function to determine agreement with the TEG and OPA assays for detecting NSAID insensitivity.

Transcellular activation has been suggested as an important clinical variable,¹⁹ and the TEG assay described in this report provides a way to monitor such interactions in clinical whole blood samples under physiological calcium conditions. The marked difference of the TEG whole blood assay and OPA in PRP for monitoring NSAID therapy in a subset of patients might prove relevant to the issue of NSAID insensitivity. It would be interesting to pursue the clinical implications of these findings in terms of differences in transcellular activation pathways developed as a consequence of chronic NSAID exposure.

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