Polyol Pathway Activation and Glutathione Redox Status in Non–Insulin-Dependent Diabetic Patients

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The current study aimed to evaluate whether nicotinamide adenine dinucleotide phosphate (NADPH) alteration in erythrocytes from patients with non–insulin-dependent diabetes mellitus (NIDDM) is responsible for the impaired glutathione (GSH) redox status, and to assess if short-term inhibition of the polyol pathway normalizes NADPH levels and GSH redox status via an amelioration of the NADPH/total NADP (tNADP) ratio. For this purpose, erythrocyte NADPH and GSH levels were measured in 18 NIDDM patients at baseline and then after 1 week of random double-blind assignment to treatment with either tolrestat (an aldose reductase inhibitor, 200 mg daily) (n = 12) or placebo (n = 6). A group of 16 healthy volunteers served as the control. In the basal condition, mean GSH ($P < .0001$) and NADPH ($P < .0001$) levels and NADPH/tNADP ($P < .0001$) and GSH/glutathione disulfide (GSSG) ($P < .005$) ratios were lower in NIDDM patients than in control subjects. Tolrestat treatment increased GSH levels ($P < .05$ *v* placebo and baseline) and the NADPH/tNADP ratio ($P < .05$ *v* placebo and baseline). Interestingly, tolrestat-induced changes in GSH and NADPH levels and in GSH/GSSG and NADPH/tNADP ratios were significant only in patients who showed a decreased NADPH/tNADP ratio at baseline (n = 8). In these latter patients, we also found a direct correlation between percentage increments in GSH levels and NADPH/tNADP ratios after tolrestat treatment ($r = .71$, $P < .05$). In conclusion, our findings support the hypothesis that polyol pathway activation decreases NADPH and GSH levels. Accordingly, short-term inhibition of this enzymatic route increased both the GSH level and the NADPH/tNADP ratio. These changes were observable only in the subgroup of patients with an abnormal NADPH/tNADP ratio at baseline. Polyol pathway inhibition could be useful for decreasing oxidative stress in NIDDM.

THE POLYOL PATHWAY is a non–insulin-dependent metabolic route for glucose utilization.[1-3] Enzymes involved in this pathway are aldose reductase, which is responsible for glucose conversion to sorbitol, and sorbitol dehydrogenase, which finally converts sorbitol to fructose.[4,5]

An increment of the circulating glucose concentration activates both of these enzymes, thereby leading to conversion of reduced nicotinamide adenine dinucleotide phosphate (NADPH) to $NADP^+$ and NAD^+ to NADH, respectively.[6,7] Therefore, hyperglycemia should overstimulate the polyol pathway and consequently increase sorbitol formation. Since sorbitol poorly penetrates the cell membrane, its accumulation has been suggested to favor osmotic stress and the development of morphofunctional abnormalities in various tissues, ie, nerve, lens, etc.[8,9]

In contrast to this theory, the more recent "redox hypothesis" suggests that chronic accumulation of $NADP^+$ and NADH and decreased supply of NADPH and NAD^+ are responsible for tissue damage due to polyol pathway overstimulation.[6,7,10-12] In particular, glutathione reductase is the main enzyme of the glutathione (γ-glutamylcysteinyl glycine [GSH]) redox cycle.[13] This enzyme requires elevated levels of NADPH to reduce glutathione disulfide (GSSG) produced during hydroperoxide metabolism. Therefore, a decrement of NADPH supply to the GSH redox cycle, which in turn should be due to hyperglycemia-related polyol pathway overstimulation, might increase oxidative stress in various tissues, thus favoring the development of diabetic complications such as neuropathy or retinopathy.[13]

In keeping with this intriguing hypothesis, an impaired function of the GSH redox cycle has already been described in non–insulin-dependent diabetes mellitus (NIDDM) patients.[14] Although no studies have evaluated the possible linkage between this abnormality and overstimulation of the polyol pathway, we have recently found that selective inhibition of aldose reductase by tolrestat treatment restored sorbitol, GSH, and the GSH/GSSG ratio to near-normal values in red blood cells (RBCs) obtained from NIDDM patients.[15] Therefore, we speculated that a tolrestat-induced increment of the NADPH supply to glutathione reductase was responsible for the observed improvement of intracellular GSH redox status.[15] Nevertheless, whether NADPH is reduced in RBCs from NIDDM patients and increases after tolrestat treatment is unknown.

To address this topic, the NADP system was evaluated in RBCs from NIDDM patients before and after 1 week of random double-blind assignment to either tolrestat or placebo treatment. To avoid any confounding factor related to age, weight, hypertension, and diabetes-related late complications, the study population was carefully selected among lean, normotensive NIDDM patients in good metabolic control and without renal, ocular, or cardiovascular complications. Since alteration of the nicotinamide dinucleotide phosphates ratio has to be considered a key element for GSH redox balance,[12] the obtained results were also evaluated by post hoc analysis, dividing patients into groups with a normal or reduced RBC NADPH/total NADP (tNADP) ratio. For this purpose, we also assessed nicotinamide dinucleotide phosphates in a selected cohort of nondiabetic subjects.

SUBJECTS AND METHODS

After provision of informed consent, 18 NIDDM outpatients (13 men and five women, aged 47 to 65 years) and 16 healthy volunteers (eight

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men and eight women, aged 38 to 65 years) of the same age range and similar gender proportion as the NIDDM patients were enrolled onto the study. Inclusion criteria for diabetic patients were as follows: (1) body mass index less than 26 kg/m^2, (2) supine systolic blood pressure less than 140 mm Hg, (3) supine diastolic blood pressure less than 90 mm Hg, (4) microalbuminuria less than 20 μg/min on three consecutive 24-hour urine collections, (5) absence of fundus oculi abnormalities, and (6) absence of concomitant diseases. All subjects were white, nonsmokers who did not drink more than 10 g of alcohol per day. NIDDM patients were in good metabolic control (ie, hemoglobin A_{1c} [HbA_{1c}] less than 7.5%, fasting plasma glucose < 7.8 mmol/L, and plasma glucose < 10.0 mmol/L 2 to 4 hours after breakfast and lunch) and under treatment with glibenclamide 5 mg twice per day. All other drugs, with special regard to those known to interfere with glutathione metabolism (ie, diethyldithiocarbamate, D-penicillamine, iodoacetate, etc.) or antioxidant agents (ie, ascorbic acid, *N*-acetyl-L-cysteine, etc.), were carefully excluded.

One week before entering the study, both patients and controls received a weight-maintaining diet containing about 50% carbohydrate, 30% protein, and 20% lipid. Then, blood samples for laboratory measurements were withdrawn from each patient and control subject at 9 AM in the fasting state. After baseline blood sampling NIDDM patients were randomly, double-blindly assigned to receive either tolrestat (200 mg once daily, n = 12) or placebo (n = 6). After 1 week of treatment, blood samples for RBC NADPH, tNADP, GSH, GSSG, and sorbitol content were taken again in both groups.

Materials

Glutathione reductase, glucose-6-phosphate dehydrogenase, NADP, NADPH, and glucose-6-phosphate were purchased from Boehringer (Mannheim, Germany). 2-Vinylpyridine was obtained from Aldrich (Steinheim, Germany). GSH, GSSG, 5-sulfosalicylic acid, cysteine HCl, 5,5′-dithiobis-2-nitrobenzoic acid, 6-phosphogluconic acid, triethanolamine, and Tris were obtained from Sigma (St Louis, MO). A test combination for hemoglobin and microalbuminuria was purchased from Boehringer.

Preparation of Samples

Heparinized venous blood samples from each subject were immediately chilled at 4°C, and 7 mL whole blood was mixed with 0.9% NaCl and layered onto Hystopaque-1077 (Sigma Chemical, St Louis, MO). After centrifugation for 30 minutes at 20°C, the plasma and buffy coat were carefully removed. Then, erythrocytes were washed three times with phosphate-buffered saline (pH 7.4), at 4°C and used for the following laboratory measurements.

Sorbitol

Packed RBCs were precipitated with cold 6% perchloric acid. Supernatants were neutralized at 4°C with K_2CO_3 and used for sorbitol determination by the enzymatic method.[16]

Glutathione

For determination of GSH levels, 0.1 mL packed RBCs were diluted with 0.1 mL isotonic saline and 0.5 mL HCl (10 mmol/L). Then, RBCs were lysed in dry-ice acetone, thawed three times, and centrifuged for 10 minutes at 4°C. Supernatants were deproteinized with 10% 5-sulfosalicylic acid and used for total glutathione determination (GSH + GSSG) by the enzymatic method described by Anderson.[17] For GSSG determination, 0.1 mL deproteinized supernatants were treated with 0.002 mL 2-vinylpyridine and neutralized with triethanolamine at a final pH of 6.5.[18] After a 60-minute incubation, the supernatants were used for GSH measurement.[17] Recovery for both procedures was 96% to 102% (mean, 98%). Interassay and intraassay variabilities were less than 10%.

Nicotinamide Dinucleotide Phosphates

Erythrocyte NADPH and tNADP concentrations were determined by the enzymatic method of Klingenberg.[19] To avoid NADPH oxidation during the assay, 0.5 mmol/L cysteine-HCl was added to packed RBCs. Recovery for both NADPH and tNADP determinations was 92% to 102% (mean, 96%). Interassay and intraassay variabilities were less than 10%.

Glucose-6-Phosphate Dehydrogenase Activity

To exclude possible subclinical defects of glucose-6-phosphate dehydrogenase activity, it was assessed by an enzymatic method.[20]

Statistical Analysis

Statistical analysis was performed using a chi-square test or Fisher's exact test for independence and paired and unpaired Student *t* tests as appropriate. Regression and correlation techniques were used to evaluate linear relationships between variables. When necessary, a logarithmic transformation was used to normalize the data, or the Wilcoxon test was used. Effects of tolrestat and/or placebo on each parameter were analyzed by ANOVA with Bonferroni protection. Unless otherwise stated, all data are presented as the mean ± SD. Statistical significance was reached at *P* less than .05.[21] All calculations were made with the computer program Stat-view II (Abacus Concepts, Berkeley, CA).

RESULTS

Compared with control subjects, diabetic patients had higher mean levels of fasting glucose, HbA_{1c}, and RBC sorbitol (Table 1). Interestingly, our data confirmed that the mean GSH concentration ($P < .0001$) and mean GSH/GSSG ratio ($P < .005$) were lower in RBCs from diabetic patients versus controls. Furthermore, NADPH paralleled the behavior of GSH levels, being 26.1% less in diabetic patients than in normal subjects ($P < .0001$). By contrast, the mean NADPH/tNADP ratio was higher in control subjects than in NIDDM patients

Table 1. Baseline Clinical and Laboratory Data for 18 Non-obese, Normotensive NIDDM Patients and 16 Control Subjects

	NIDDM Patients		Control Subjects	
Variable	Mean ± SD	Range	Mean ± SD	Range
Age (yr)	56 ± 5	47-65	50 ± 7	38-65
Fasting glucose (mmol/L)	6.58 ± 0.48	5.9-7.7	4.65 ± 0.7*	4.3-5.0
HbA_{1c} (%)	7.01 ± 0.22	6.7-7.4	4.48 ± 0.50*	4.2-4.7
RBC sorbitol (nmol/g Hb)	26.33 ± 2.29	21.50-30.51	11.27 ± 5.5*	3.32-19.01
RBC GSH (μmol/g Hb)	5.6 ± 0.8	5.2-6.1	7.6 ± 1.0*	7.0-8.1
RBC GSSG (μmol/g Hb)	0.61 ± 0.30	0.41-1.6	0.26 ± 0.08*	0.21-0.30
GSH/GSSG ratio	10.51 ± 3.70	3.2-19.9	15.94 ± 5.43†	9.6-29.4
RBC tNADP (nmol/g Hb)	129.98 ± 26.5	84-159.4	121 ± 15	113.4-129.2
RBC NADPH (nmol/g Hb)	96.78 ± 20.6	55.7-129.0	121 ± 14*	113.7-129.0
NADPH/ tNADP ratio	0.71	0.50-1.1	0.99*	0.82-1.3

*$P < .0001$.
†$P < .005$.

($P < .0001$). No other statistically significant differences were found between the two groups. In particular, patients and controls did not significantly differ in age, gender distribution, blood pressure, and serum creatinine. Erythrocyte tNADP concentration was higher in diabetic patients than in normal subjects, but the difference was not statistically significant (ANOVA: F = 1.39, $P = .24$, NS).

With regard to the effects of tolrestat on the single components of GSH redox cycle and sorbitol in RBCs, 1 week of treatment induced normalization of mean sorbitol levels. Indeed, baseline erythrocyte sorbitol concentrations were 3.3 to 19 nmol/g Hb in control subjects and 21.5 to 30.5 nmol/g Hb in NIDDM patients. After NIDDM patients (n = 12) were treated with tolrestat, mean sorbitol levels decreased from 26.0 ± 2.55 nmol/g Hb (range, 21.5 to 30.5) to 10.69 ± 2.71 nmol/g Hb (range, 6.9 to 15.6; $P < .0001$). By contrast, sorbitol levels did not change in NIDDM patients (n = 6) treated with placebo.

As previously demonstrated,[15] erythrocyte GSH content increased after tolrestat treatment ($P < .05$ *v* baseline or placebo), whereas it remaining unchanged in the placebo group. In contrast, GSSG levels were mildly affected by both tolrestat (from 0.63 ± 0.55 to 0.55 ± 0.18 μmol/g Hb, $P = .46$, NS) and placebo. As a consequence, the mean GSH/GSSG ratio was not significantly improved by active treatment ($P = .13$, NS).

Statistical assessment of treatment effects on nicotinamide dinucleotides showed a significant decrement of tNADP in the tolrestat group (from 134.6 ± 29.0 to 114.2 ± 16.8 nmol/g Hb, $P < .05$), but not in the placebo group. Although NADPH increased in significantly in tolrestat-treated patients (from 102.8 ± 16.8 to 110.6 ± 24.8, $P = 0.2$, NS), due to the marked changes of tNADP, the mean NADPH/tNADP ratio was significantly improved by tolrestat therapy (from 0.75 ± 0.18 to 0.95 ± 0.14, $P < .05$).

Since an abnormality of the NADPH/tNADP ratio might deeply influence the GSH redox cycle and, as a consequence, the effect of tolrestat treatment, the data were also analyzed after patients were divided according to baseline NADPH/tNADP ratio. Compared with the range of normality (0.82 to 1.3; Fig 1A) in the active treated group, eight NIDDM patients had a low range (0.50 to 0.76) and four had a normal range (0.90 to 1.11) for the NADPH/tNADP ratio (Fig 1A). The same ratio was subnormal in four and normal in two patients of the placebo group. Tolrestat treatment reduced sorbitol levels in all patients (Fig 1B). By contrast, GSH levels increased after the same therapy only in patients with a reduced NADPH/tNADP ratio at baseline ($P < .05$; Fig 1C). Interestingly, the same ratio increased to normal after tolrestat treatment in seven of eight patients in whom it was basally reduced, while remaining unchanged in four tolrestat-treated patients with a normal NADPH/tNADP ratio at baseline. All of these variables were unaffected by placebo treatment. In the subgroup of patients with a reduced NADPH/tNADP ratio, percentage changes in the NADPH/tNADP ratio and GSH levels after tolrestat treatment were always directly correlated ($r = .71$, $P < .05$; Fig 2).

DISCUSSION

Herewith, we demonstrate that the mean NADPH/tNADP ratio is reduced in erythrocytes from lean, normotensive NIDDM patients in good metabolic control, and that short-term inhibition of the polyol pathway by tolrestat treatment normalizes the same ratio. Considering each individual, we also show that the NADPH/tNADP ratio was less than the range of normality in 12 of 18 NIDDM patients (75%; Fig 1), eight of whom were randomly, double-blindly assigned to receive the

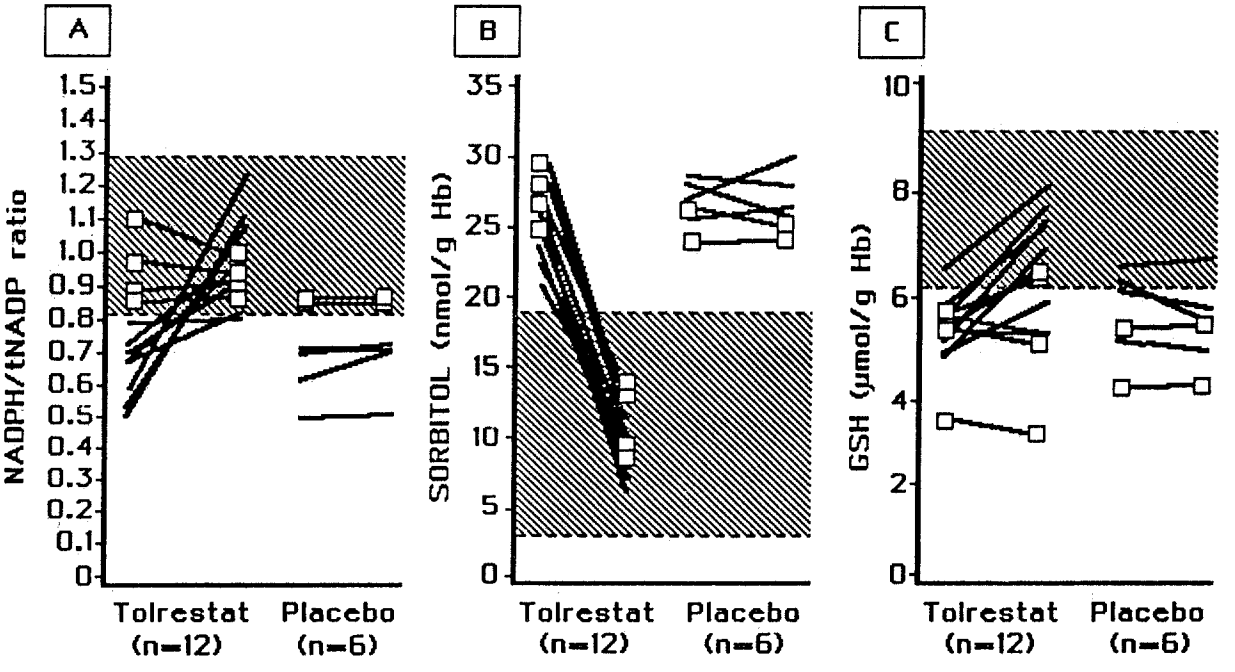

Fig 1. Changes in RBC NADPH/tNADP ratio (A), sorbitol level (B), and GSH concentration (C) in 18 non-obese, normotensive patients with NIDDM before and after 1 week of tolrestat or placebo treatment. Shaded areas indicate the range of normality for each parameter, as detected in a group of 16 age-, sex-, and weight-matched healthy volunteers.

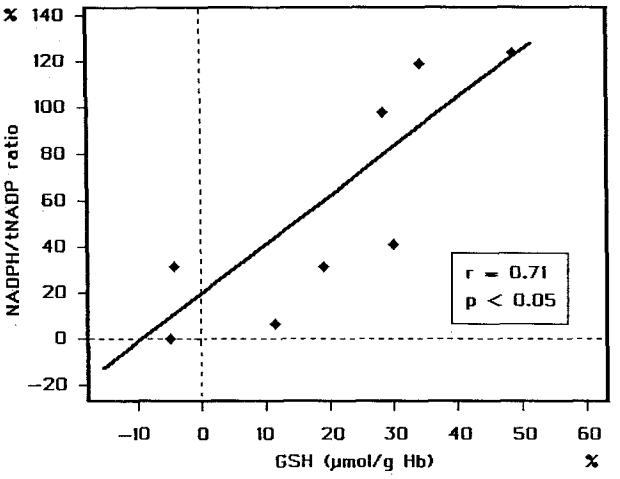

Fig 2. Correlation between percentage changes in GSH level and NADPH/tNADP ratio after 1 week of tolrestat treatment in 8 NIDDM patients with a subnormal NADPH/tNADP ratio before therapy.

active treatment, ie, tolrestat, and four the placebo treatment. Interestingly, tolrestat normalized the above ratio in seven of eight of these patients. By contrast, tolrestat treatment did not affect the NADPH/tNADP ratio in the four active-treatment patients who had a normal ratio at baseline.

In this context, nicotinamide dinucleotides, ie, NAD^+ and NADPH, and nicotinamide dinucleotide phosphates, ie, $NADP^+$ and NADPH, are the most important hydrogen-transferring coenzymes for several dehydrogenases. In particular, NADPH mainly derives from the pentose phosphate shunt.[22] As is known, it is used for the biosynthesis of steroids and fatty acids and enters many enzymatic routes such as the polyol pathway.[22] In particular, NADPH is used in this pathway for electron transport during glucose conversion to sorbitol. Since NADPH is transformed to $NADP^+$ during this reaction, the NADPH/tNADP ratio is usually considered the indicator of the redox balance of this coenzyme.[23]

In this regard, NADPH is also used by glutathione reductase to convert GSSG to GSH. Therefore, a decreased supply of NADPH to the GSH redox cycle could be responsible for an alteration of GSH levels and the consequent increment of oxidative stress. Since NADPH is oxidized to NADP by the polyol pathway, an excessive activity of this enzymatic route due to NIDDM should induce a decrease of NADPH levels. According to this hypothesis, the mean NADPH/tNADP ratio was significantly decreased in our NIDDM patients. Similarly, the role of polyol pathway activation in reducing NADPH levels is also supported by the significant increment of this ratio observed in the same patients after 1 week of tolrestat treatment. Therefore, it can be speculated that polyol pathway activation may facilitate diabetes-related organ damage by increasing the oxidative stress rather than sorbitol levels. In contrast to this interpretation, although all patients had increased sorbitol levels at baseline (Fig 1), thereby indicating an overstimulation of the polyol pathway, four of 12 tolrestat-treated patients showed a normal NADPH/tNADP ratio, which was unaffected by tolrestat treatment. Thus, polyol pathway inhibition seems to modulate NADPH supply only in some NIDDM patients, namely in those with a subnormal NADPH/tNADP ratio.

In terms of study limitations, the normal NADPH/tNADP ratio could be maintained in some NIDDM patients by other enzymatic routes such as the pentose phosphate pathway.[22] Unfortunately, we did not investigate this pathway in our patients, and we can only suggest such a hypothesis. Similarly, we are aware that a larger number of patients with a normal NADPH/tNADP ratio at baseline might result in a definite increment of the ratio after tolrestat treatment. In this regard, interesting data could be obtained in tolrestat-treated normal subjects, but ethical concerns made it impossible for us to obtain a group of tolrestat-treated healthy volunteers. Finally, we have not evaluated RBC membrane permeability, an increase of which has been suggested to contribute to the altered redox status in NIDDM patients.[24] By contrast, only patients with normal RBC glucose-6-phosphate dehydrogenase activity were recruited. Thus, the possible influence of a decreased activity of this enzyme on GSH levels in RBCs from NIDDM patients cannot represent a bias of our study.[25]

Another interesting finding of our research is the increment of GSH concentration observed after tolrestat treatment. In this regard, a reduced GSH level and GSH/GSSG ratio have already been demonstrated in diabetic patients, and interpreted as a clear demonstration of increased oxidative stress.[15,26-29] Indeed, glutathione is a well-known antioxidant agent.[13] In particular, a suitable GSH/GSSG ratio is required for cellular protection against free radicals. The present study provides important information in this area, suggesting that the reduced GSH levels are due to polyol pathway overactivity and the consequent decrement of NADPH supply to the GSH redox cycle. Accordingly, tolrestat treatment simultaneously increased both the GSH level and NADPH/tNADP ratio. To further support such a hypothesis, we found a direct correlation between percentage changes of GSH and the NADPH/tNADP ratio after 1 week of tolrestat treatment.

In contrast to our results, treatment of diabetic rats with another aldose reductase inhibitor, ie, sorbinil, failed to influence GSH concentration in peripheral nerves.[28] In this regard, the discrepancy between human and rat data could derive from a different susceptibility to oxidative stress between nerves and erythrocytes. In keeping with this explanation, RBCs and lenses[29] are supplied by the GSH redox cycle and pentose phosphate shunt, both connected to the polyol pathway to remove NADPH from GSH regeneration. By contrast, the GSH redox cycle and pentose phosphate shunt are poorly represented in nerves.[29]

In conclusion, the present randomized, double-blind study provides evidence that an alteration of nicotinamide dinucleotide phosphates is responsible for reduced GSH levels in NIDDM patients. Furthermore, our findings strongly support the hypothesis that polyol pathway inhibition could reduce oxidative stress in diabetic patients. Whether such treatment represents a suitable tool to preserve the cellular redox status in NIDDM patients should be verified by large-scale clinical trials.

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