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Acute Phase Immune Response to Exercise Coexists with Decreased Neutrophil Antioxidant Enzyme Defences

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Long-duration or damaging exercise initiates reactions that resemble the acute phase response to infection and induces neutrophil priming for oxidative activity. Our objective was to establish the status of the antioxidant defences and of the oxidative equilibrium in the neutrophils of sportsmen prior to and after intense physical exercise. Nine voluntary male professional cyclists participated in this study. The exercise was a cycling mountain stage (171 km) and the cyclists took a mean ± SEM of 270 ± 12 min to complete it. We determined the activities of catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx), the levels and activity of superoxide dismutase (SOD), the concentrations of ascorbate, glutathione and glutathione disulphide (GSSG) and DNA levels in neutrophils. The cycling stage decreased enzyme activities expressed per DNA units: CAT (33%), SOD (38%), GPx (65%); increased ascorbate concentration in neutrophils and decreased the GSH/GSSG ratio and the enzyme activities expressed per DNA units. Neutrophils could contribute to plasma antioxidant defences against oxidative stress induced by exercise because they probably provide antioxidant enzymes and ascorbate.

Keywords: Neutrophil; Exercise; Oxidative stress; Antioxidants

INTRODUCTION

Long-duration or damaging exercise induces oxidative stress[1,2] and often initiates reactions that resemble the acute phase response to infection,[3] which is modulated and sustained through the action of cytokines, notably interleukin IL-1, IL-6 and tumour necrosis factor (TNF),[3] resulting in an inflammatory response.[4] It has been suggested that this inflammation-like immune response is necessary for muscular regeneration and adaptation to physical exercise[5,6] (although plasma levels of several cytokines increase independent of muscle damage[7,8]) but also that strenuous physical exercise can increase the risk for upper respiratory tract infections.[9]

Circulating leukocytes represent a population of cells on their way towards participation in ongoing tissue surveillance, repair and adaptation. Exercise induces neutrophil priming for oxidative activity determined by luminol-dependent chemiluminescence.[10] The activated neutrophil presents increased capabilities to synthesise reactive oxygen species (ROS), as evidenced by the high activity of NADPH-oxidase[11] but this increased oxidative activity can compromise host defences through auto-oxidative inhibition of neutrophil functions and inhibition of the proliferation of lymphocytes and natural killer cells.[12,13] The machinery to prevent auto-oxidative processes involves the superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) enzymes and also the antioxidant action of vitamin C, vitamin E and glutathione.[14] Ascorbate, a potent antioxidant vitamin, is found in normal circulating human neutrophils in millimolar concentration[15] and it is described that the neutrophil can operate by recycling ascorbate from its oxidative product dehydroascorbate. Neutrophils preferentially uptake

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dehydroascorbate from the circulation, then it is reduced to ascorbate by a glutathione-independent system and is accumulated in the neutrophil.[16] From this point of view, neutrophils may also contribute to decrease the oxidative stress status because recycling rapidly provides high concentrations of ascorbate for intracellular oxidant quenching. The effects of endurance exercise on stress hormones, cytokines, neutrophil dynamics, cell activation, cell adhesion, cellular memory function of the immune system and on muscle damage[9,10,17] are well documented. However, the adaptations to intense physical activity of the antioxidant enzymes of neutrophils and other immune cells remain unknown. The contribution of recycling ascorbate system on the installation of oxidative stress associated to intense physical exercise is yet unknown. Thus, it may be of interest to understand some processes such as muscle repair and inflammation and upper respiratory tract infections in which the genesis and function of ROS are important tools.

Thiol redox cycles play central roles in the antioxidant defence network.[18,19] Blood glutathione homeostasis has been suggested as a determinant of resting and exercise-induced oxidative stress in young men.[20] Oxidation of thiol to disulphides is a sensitive marker of oxidative stress.[21,22] Studies involving human blood GSH oxidation during exercise are limited, but previous studies show that exhaustive exercise in rats remarkably increases glutathione disulphide (GSSG) concentrations in plasma.[23] At the moment studies involving glutathione determinations in neutrophils during exercise are unknown.

The intention of this study was to establish the status of the antioxidant defences and of the oxidative equilibrium in neutrophils of sportsmen prior to and after very intense physical exercise such as a cycling stage. The period studied was the same as others claim to be the acute phase immune response period. We have previously evidenced adaptations to intense physical exercise of the antioxidant enzyme in erythrocytes as a result of enzyme activation induced by oxidative stress.[24] In the present work, we show the occurrence of low activities of antioxidant enzymes and glutathione and high levels of ascorbate in neutrophils after a mountain cycling stage, during the acute phase immune response to exercise.

MATERIALS AND METHODS

Subjects and Exercise

Nine voluntary male subjects participated in this study. They were all professional cyclists participating in the "Setmana Catalana 2000", a five-day competition for professional cyclists near Barcelona (Spain). Subjects were informed of the purpose of this study and the possible risks involved before giving their oral consent to participate. The study protocol was in accordance with the Declaration of Helsinki and was approved by the local ethics committee (Ethical Committee of "Hospital Son Dureta").

The exercise was a mountain stage (171 km) of the "Setmana Catalana 2000". This mountain cycling stage was the third stage of the "Setmana Catalana 2000". The two prior stages were shorter, 120 and 150 km, and without mountain. The mean age of the sportsmen ($\pm$SEM) was 23.8 ± 0.9 years, height 180 ± 2 cm, and weight 70.0 ± 1.5 kg. The values were in the range of normal subjects with the same age. Their VO_2max was $80.2 \pm 1.6\,ml\,kg^{-1}\,min^{-1}$, which was higher than that of normal subjects. The cyclists took a mean $\pm$ SEM of 270 ± 12 min to complete this stage.

Experimental Procedure

Venous blood samples were taken from the antecubital vein with suitable vacutainers. The basal venous blood was obtained from the subjects participating in this study on the morning of the cycling stage day, after 12 h overnight fasted conditions; immediately after the stage; and 3 h after finishing the stage. We also obtained a blood sample on the morning of the next day, once again after 12 h overnight fasted conditions, which represents approximately 15 h of recovery.

A blood vacutainer was used to purify neutrophils. The enzyme activities of CAT, glutathione reductase (GR), GPx and the levels and activity of SOD were determined in these neutrophils. We determined DNA levels in the final cellular suspension in order to evaluate the pattern of the changes in the number of circulating neutrophils. We also determined concentrations of vitamin C, glutathione and GSSG in neutrophils.

Neutrophil Purification

Blood samples were obtained from the antecubital vein of sportsmen after overnight fasting in suitable vacutainers with EDTA as anticoagulant and were used to purify the neutrophil fraction following an adaptation of the method described by Boyum.[25] A known blood volume was centrifuged at 900*g*, 4°C for 30 min after carefully introducing on Ficoll in a proportion of 1.5:1. The plasma, the lymphocyte phase and the organic one were discarded. The phase at the bottom contained the erythrocytes and neutrophils. Erythrocytes were haemolysed with 50 ml of ammonium chloride 0.15 M at 4°C.

The suspension was centrifuged at 750*g*, 4°C for 15 min and then the supernatant was discarded. The neutrophil phase at the bottom was washed first with 50 ml of ammonium chloride 0.15 M and then with 50 ml of PBS. The slurry was centrifuged again as above. Finally, neutrophils were lysed with distilled water (1:10). The neutrophils' suspension obtained from a known blood volume was used to determine antioxidant enzyme activities, DNA, glutathione and vitamin C concentrations and, finally, SOD levels.

Neutrophil DNA Determination

DNA from neutrophils was determined by a fluorimetric method. In order to hydrolyse the neutrophil RNA, NaOH was added to suitable volumes of the neutrophil suspensions. Then the samples were neutralised with HCl and diaminobutyric acid was added. After that, the samples were incubated for 50 min at 60°C in order to allow the reaction of DNA with DABA. Finally, each sample was acidified with HCl and the fluorescence was determined with an excitation wavelength of 405 nm and an emission wavelength of 505 nm. We used DNA from salmon testes (Sigma) as standard for the determination of DNA concentrations.

The neutrophil DNA determination method was applied to the isolated neutrophils from a known blood volume. Then, the DNA quantificated in these samples could be assigned to the expression of DNA per ml of blood.

Enzymatic Determinations

We determined the activities of SOD, CAT, GPx and GR in the neutrophils. All activities were determined with a Shimadzu UV-2100 spectrophotometer at 37°C.

Catalase activity was measured by the spectrophotometric method of Aebi[26] based on the decomposition of H_2O_2.

Glutathione reductase activity was measured by a modification of the Goldberg and Spooner[27] spectrophotometric method. This assay required oxidised glutathione as the substrate.

Glutathione Peroxidase activity was measured using an adaptation of the spectrophotometric method of Flohé and Gunzler.[28] This assay required H_2O_2 as a substrate and GR and NADPH as enzyme indicators.

Superoxide dismutase activity was measured in neutrophil preparation by an adaptation of the method of McCord and Fridovich.[29] The xanthine/xanthine oxidase system was used to generate the superoxide anion. This anion produced the reduction of cytochrome C, which was monitored at 550 nm. The SOD of the sample removed the anion superoxide and produced an inhibition of the reduction. The value of this reduction was used as a measure of the enzyme activity.

ELISA Determination of Superoxide Dismutase (SOD)

The Cu/Zn SOD levels were determined by ELISA using monoclonal antibody Anti SOD (mouse IgG1 isotype, Sigma) which recognises human SOD, following an adaptation of the method to determine the uncoupling protein.[30]

Suitable dilutions of the neutrophil suspensions (50 μl) were placed in each well of the plate (Polystyrene Assay Plate, Costar). The plate was then incubated (37°C for 3 h) in order to adsorb the sample proteins to the surface of each well. A solution of 1% bovine albumin (100 μl) was added into each well and the plate was incubated (37°C for 3 h) in order to saturate all binding protein sites of the plate. After that the plate was emptied and each well was washed four times with NaCl 0.9%-Tween 20. The commercial monoclonal antibody (100 μl diluted 1000 folds with PBS-Tween 20) was placed into each well and the plate was newly incubated at 37°C. After 3 h, the plate was emptied and each well was washed as above. The secondary antibody against the IgG chain, conjugated to alkaline phosphatase (100 μl diluted 500 fold) was placed into each well and the plate was incubated in the same conditions as the other times. The wells were newly washed and the phosphatase substrate solution (100 μl) was added to each well. The plate was finally incubated for 90 min at 37°C and the absorbance was measured at 405 nm. We used Cu/Zn SOD from human erythrocytes (Sigma) as the patron to calibrate the method. We observed a linear correlation ($r = 0.995$) between the concentration of SOD present in the well and the absorbance measured at 405 nm. This ratio was used to calculate the SOD concentrations in the samples.

Neutrophil Vitamin C Determinations

Neutrophil vitamin C was determined by an HPLC method with electrochemical detection.[31,32] Immediately after neutrophil purification, neutrophil suspensions were deproteinised with 30% trichloroacetic acid containing 2 mM EDTA. After complete precipitation of the proteins, the samples were centrifuged for 5 min at 15,000*g* at 4°C. Deproteinised samples were stored at −70°C for a week and were then analysed. Appropriate volumes of deproteinised neutrophil suspensions, previously diluted 1:1 with distilled water, and were injected into the HPLC system. The mobile phase consisted of 0.05 M sodium phosphate, 0.05 M sodium acetate, 189 μM dodecyltrimethylammonium chloride and 36.6 μM tetraoctylammonium bromide in

25/75 methanol/water (v/v), pH 4.8. The HPLC system was a Shimadzu with a Waters Inc electrochemical detector and a Nova Pak, C18, 3.9 mm × 150 mm column. The potential of the chromatographic detector was set at 0.7 V versus an Ag/AgCl reference electrode.

Glutathione Determinations in Neutrophils

Glutathione (GSH) and GSSG were determined in neutrophils by an adaptation of the method described by Spitz *et al.*[33] This method determines glutathione and other thiols by reverse-phase high performance liquid chromatography by forming fluorescent derivatives with *N*-(1-Pyrenyl)maleimide (NPM).

Neutrophils were deproteinised using the same method as above. Then, two aliquots of 250 µl of the acidic supernatant were taken and neutralised with $KOH/NaHCO_3$ 2 N. One aliquot was used to determine GSH. This aliquot was derivatised by the addition of NPM. Derivatisation was completed within 5 min and the fluorescent derivatives were then stabilised by acidification. At this point the sample was injected into the HPLC system. In order to determine GSSG, 2-vinylpyridine was added to the second aliquot and the mixture was incubated at room temperature for 60 min to block free GSH. Then GSSG was reduced enzymatically to GSH by addition of NADPH and GR in a very fast process. An aliquot was immediately taken out to measure GSH according to the procedure given above.

The HPLC was a Shimadzu with a fluorescent detector operating at an excitation wavelength of 330 nm and an emission wavelength of 380 nm. The column was a Waters Spherisorb 5 µm ODS2, 34.6 mm × 150 mm. Mobile phase consisted of 650: 350 acetonitrile:H_2O acidified with 1 ml per litre of acetic acid and 1 ml per litre of *ortho*-phosphoric acid.

Statistical Analysis

Statistical analysis was carried out using a statistical package for social sciences (SPSS 9 for windows). All the data were tested for their normal distribution. Results are expressed as means ± SEM and $P < 0.05$ was considered statistically significant. ANOVA for repeated measures was used to determine the significance of the differences in all the parameters determined during the stage and the recovery.

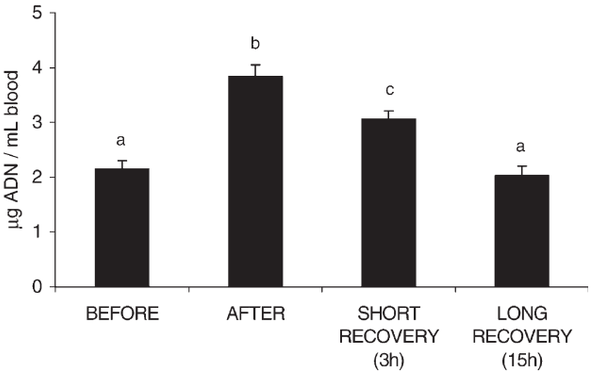

FIGURE 1 Changes in neutrophils' DNA during stage and recovery. The presence of letters indicates a significant ANOVA for repeated measures. Different letters indicate significantly different values ($P < 0.05$).

RESULTS

The cycling stage significantly increased the DNA concentrations (79%) in the neutrophil fraction of blood (Fig. 1). After 3 h of recovery, neutrophil DNA levels decreased, although they remained significantly higher than the basal ones and returned to the basal levels after 15 h of recovery (on the morning of the following day).

Antioxidant enzyme activities were expressed per DNA units (Table I). This expression refers the enzyme activity to a cellular basis. The mountain cycling stage induced a general decrease in the antioxidant enzyme activities of neutrophils and the return to basal levels during recovery depended on the particular enzyme. The CAT activity expressed on a neutrophil DNA basis decreased about 33%, the SOD activity decreased 38%, and GPx decreased 65%. Neutrophil GR decreased only 17% but this decrease was not statistically significant and quickly attained the basal levels after a short period of recovery. Neutrophil CAT and SOD slowly increased their levels during recovery until reaching the basal levels after 15 h. Neutrophil GPx maintained low levels even 15 h after the end of the stage (Table I).

The changes described in SOD activity in neutrophils were the same as those experienced

TABLE I Activities of neutrophil antioxidant enzymes during a cycling stage and recovery

	Before	After	Short recovery (3 h)	Long recovery (15 h)
Catalase (k/µg DNA)	0.09 ± 0.01^a	0.06 ± 0.01^b	0.07 ± 0.01^b	0.10 ± 0.01^a
Glutathione peroxidase (nkat/µg DNA)	0.37 ± 0.06^a	0.13 ± 0.02^b	0.16 ± 0.01^b	0.19 ± 0.02^b
Glutathione reductase (nkat/µg DNA)	0.12 ± 0.01	0.10 ± 0.01	0.12 ± 0.02	0.13 ± 0.02
SOD (pkat/ng DNA)	31.0 ± 3.0^a	19.1 ± 2.2^b	25.4 ± 2.0^a	34.4 ± 4.1^a

The letters in superscript indicates a significant ANOVA for repeated measures. Different letters indicate significantly different values ($P < 0.05$).

TABLE II Changes in protein concentration and specific activity of neutrophil SOD during a cycling stage and recovery

	Before	After	Short recovery (3 h)	Long recovery (15 h)
μg SOD/μg DNA	13.0 ± 0.8[a]	6.71 ± 0.55[b]	8.38 ± 0.94[b]	13.4 ± 1.3[a]
pkat/μg SOD	2.49 ± 0.17	2.89 ± 0.14	3.02 ± 0.22	2.83 ± 0.28

The letters in superscript indicates a significant ANOVA for repeated measures. Different letters indicate significantly different values ($P < 0.05$).

by the SOD levels determined by ELISA (Table II). The specific activity of SOD (expressed as pkat/μg of SOD) were unchanged during the cycling stage and recovery and, then, the decrease observed in the SOD activity per DNA units after cycling stage were also observed in the SOD protein levels.

The cycling stage increased ascorbate concentration in neutrophils (Table III), expressed per DNA units (125%). This high ascorbate level per DNA units was maintained in neutrophils during short-recovery; then ascorbate decreased their levels, during the long recovery until the following morning. The ascorbate levels present in neutrophils 15 h after the mountain cycling stage were the same as those presented before the stage. The total glutathione concentration expressed with respect to DNA units decreased in neutrophils about 43% after the cycling stage. These low levels in neutrophils were maintained during recovery until the following morning, when cyclists presented the initial basal values again. Reduced glutathione presented a similar pattern as total glutathione. Neutrophil GSSG expressed on a DNA basis decreased significantly after the stage, and was kept lower during the short recovery, attaining again the initial basal value 3 h after the stage. The GSH/GSSG ratio decreased about 17% after the stage and returned to the initial basal values after 3 h of recovery.

DISCUSSION

The DNA content increases after physical activity in the neutrophil fraction of blood, indicating an increase in circulating neutrophils as has been pointed out in some competitions.[34–37] The neutrophilia present after intense exercise has been related with the existence of an acute phase immune response induced by exercise similar to the one induced by infection.[3] Indirect evidence reflecting the activation of neutrophils by exercise has been pointed out[38–40] after different types of exercise. However, some authors have pointed out that these plasma-increased levels are a result of the neutrophilia and do not reflect the real neutrophil activation because no changes were observed when the plasma levels were expressed per neutrophil.[10] The acute phase immune response induces the priming neutrophils to oxidative burst when they are stimulated[1] and their contribution to the oxidative stress induced by exercise has been recently evidenced.[1] Exercise induces neutrophil priming for oxidative activity, probably as part of the adaptation to exercise, but we evidence now that intense exercise also produces an important decrease in antioxidant defences of neutrophils. The neutrophil activities of SOD, CAT and GPx decrease as a consequence of the intense physical activity. The loss of antioxidant enzymes of neutrophils is in accordance with the high superoxide neutrophil production observed *in vitro* in neutrophils after downhill running.[41] It is worth noticing the lower GPx activity after 15 h of recovery, indicating a low capability to repair, with the use of glutathione as antioxidant, peroxidised neutrophil membranes from intense exercise.

The decreased enzyme antioxidant activities could be attributed to several causes. The first is the possibility of a large influx into the blood circulation of new neutrophils with lower antioxidant concentrations as a consequence of exercise. In fact, it has been shown that the antioxidant enzymes SOD, GPx and CAT were all two- to three-fold higher in aerobic alveolar macrophages than in hypoxic peritoneal macrophages.[42] In the same way, circulating neutrophils in an aerobic environment could present higher activities of antioxidant enzymes than the ones influxed from hypoxic environments. The second, as a result of the partial inactivation of the enzymatic protein due to protein modifications produced by ROS or other factors. This option can be discarded because the specific activity

TABLE III Changes in neutrophil vitamin C and glutathione during a cycling stage and recovery

	Before	After	Short recovery (3 h)	Long recovery (15 h)
Vitamin C (μg/mg DNA)	94.0 ± 6.5[a]	212 ± 12[b]	227 ± 16[b]	98.8 ± 10.9[a]
Total glutathione (μmol/mg DNA)	1.15 ± 0.08[a]	0.63 ± 0.03[b]	0.79 ± 0.04[b]	1.22 ± 0.10[a]
GSH (μmol/mg DNA)	1.05 ± 0.07[a]	0.57 ± 0.03[b]	0.72 ± 0.04[b]	1.12 ± 0.05[a]
GSSG (nmol/mg DNA)	48.9 ± 5.0[a]	31.4 ± 1.7[b]	35.3 ± 2.3[b]	51.8 ± 4.9[a]
GSH/GSSG	22.2 ± 1.5[a]	18.4 ± 1.1[b]	20.6 ± 1.2[a]	22.0 ± 1.1[a]

The letters in superscript indicates a significant ANOVA for repeated measures. Different letters indicate significantly different values ($P < 0.05$).

of SOD is maintained throughout the study. The immune-reactive SOD decreased parallel to its activity and the specific activity of this catalytic protein was maintained throughout the period studied. The third, as a result of a change in the enzyme turnover with a decreased enzyme synthesis rate or increased enzyme degradation rate. The regulation of antioxidant enzyme synthesis in immune cells is participated by ROS and cytokines.[43] The changes described during the acute phase immune response induced by exercise indicate an increase in IL-1 and several hormones[41] that could induce the antioxidant enzyme synthesis in neutrophils, even in the new cells that could have arrived to the blood circulation. Finally, the loss of antioxidant enzymes of neutrophils could be attributed to another cause: the possible secretion of these enzymes to plasma from neutrophils. The quick decrease in the antioxidant enzyme concentration in neutrophils and the distinct degree of loss observed by the different enzymes leads us to speculate on the operation of specific mechanisms to regulate the presence of these enzymes in neutrophils after intense exercise. It has been established that monocytes and macrophages can secrete arginase in order to increase the availability of intracellular arginine for iNOS and NO synthesis.[44] In the same way, we could suggest an antioxidant enzyme secretion from neutrophils after intense physical exercise. The physiological significance of the antioxidant enzymes in plasma is in study, but ecSOD is used as an index of oxidative stress.[45]

The contribution of neutrophils to the defence of ROS has been pointed out as a mechanism to recycle ascorbate and efflux it to neutralise ROS localised near the neutrophil.[3] The ascorbate concentration in neutrophils is higher than in plasma and is approximately between 1 and 1.4 mM.[30] The neutrophil ascorbate concentration in basal conditions, calculated from our results, and taking into account the neutrophil number (approximately 3000 neutrophils/μl of blood) and their volume (approximately 30 μl/10^8 neutrophils),[16] is 1 mM. It has been indicated that the plasma ascorbate concentration increases about 1.3 times immediately after a 21 km running race.[46] The neutrophil ascorbate increases its concentration about 2.5 times after the stage and during post-exercise, maintaining a positive concentration gradient to efflux ascorbate to plasma. We point out that the ascorbate increase in plasma could be partially attributed to ascorbate recycling and efflux from neutrophils induced by exercise, although the contribution of other cellular or tissue sources such as the adrenal glands has also been indicated.[46]

The effects of intense exercise on antioxidant defences of neutrophils could be evidenced by a significant decrease in the GSH/GSSG ratio after exercise but the oxidative stress was neutralised during short recovery because this index was normalised after 3 h of recovery.

Glutathione is the main intracellular antioxidant; it is synthesised from glutamine, glycine and cysteine with hydrolysis of ATP. The low glutathione content of neutrophils determined in the present paper is in accordance with that described by others.[47] The neutrophilia associated to exercise is characterised by a significant decrease in neutrophil concentration of total glutathione, which is kept low during the short-recovery period. Exhaustive exercise induces a plasma decrease of glutamine[48] and some essential amino acids.[49] The decrease in neutrophil total glutathione could be produced as a consequence of a decreased availability of its precursors for glutathione synthesis as a consequence of the energy demands of exercising muscle.

In summary, the metabolic adaptation of neutrophils induced by intense physical exercise such as a mountain cycling stage is characterised by a decrease in intracellular total glutathione, GSH/GSSG ratio and antioxidant enzyme activities, and also by an increase in intracellular ascorbate. Neutrophils could contribute to plasma antioxidant defences because they probably provide both antioxidant enzymes and ascorbate. The primed neutrophils, in order to participate in the mechanisms of muscle repair, have low enzyme antioxidant defences.

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