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ORIGINAL CONTRIBUTION

Effect of polyunsaturated fatty acids on the reactive oxygen and nitrogen species production by raw 264.7 macrophages

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Abstract
Background Polyunsaturated fatty acids (PUFAs) can affect various functions of the immune system including inflammatory responses. An oxidative burst of phagocytes accompanied by reactive oxygen species (ROS) and reactive nitrogen species (RNS) formation is one of the phagocyte functions that could be modulated by PUFAs.
Aim of the study To investigate the effects of ω-3 (α-linolenic, docosahexaenoic, eicosapentaenoic) and ω-6 (arachidonic, linoleic) PUFAs on lipopolysaccharide (LPS)-stimulated ROS and RNS production by the murine macrophage cell line RAW 264.7.
Methods Murine peritoneal macrophages RAW 264.7 were stimulated with LPS (0.1 μg/ml) and treated with 0.1–100 μM ω-3 or ω-6 PUFAs for either 8 (ROS production) or 20 h (RNS production). The cytotoxicity of PUFAs was evaluated by an ATP (adenosine triphosphate) test after both 8 and 20 h of treatment with PUFAs. Changes in ROS production by LPS-treated macrophages subsequently activated with phorbol myristate acetate (PMA) or opsonized zymosan particles (OZP) were determined by luminol-enhanced chemiluminescence, whilst the production of RNS was determined as the concentration of nitrites in cell supernatants (Griess reaction). Changes in inducible nitric oxide synthase (iNOS) expression were evaluated by Western blot analysis. The antioxidant properties of PUFAs were tested by TRAP (total peroxyl radical-trapping antioxidant parameter) assay.
Results All PUFAs in 100 μM concentration except eicosapentaenoic acid decreased ROS production. The effect was most significant when docosahexaenoic acid was used. Arachidonic acid decreased PMA-activated ROS production even in 1 and 10 μM concentrations. On the other hand, 10 and 100 μM eicosapentaenoic acid potentiated ROS production. As concerns RNS production, all the fatty acids that were tested in a concentration of 100 μM decreased iNOS expression and nitrite accumulation. Fatty acids had no significant effect on the viability and proliferation of RAW 264.7 cells. The TRAP assay confirmed that none of the tested PUFAs exerted any significant antioxidant properties.
Conclusion High concentrations of PUFAs of both ω-3 and ω-6 groups can inhibit ROS and RNS formation by stimulated macrophages. The expression of iNOS can also be inhibited. This effect, together with the absence of antioxidant activity and cytotoxic properties, indicates that PUFAs can participate in the regulation of enzymes responsible for reactive species production.

Keywords Polyunsaturated fatty acids · Reactive oxygen species · Reactive nitrogen species · RAW 264.7 cells · Chemiluminescence

Abbreviations

AA	Arachidonic acid
ALA	α-Linolenic acid
ATP	Adenosine triphosphate
CL	Chemiluminescence
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
iNOS	Inducible nitric oxide synthase
LA	Linoleic acid

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LPS	Lipopolysaccharide
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NO	Nitric oxide
OZP	Opsonized zymosan particles
PBS	Phosphate buffered saline
PMA	Phorbol myristate acetate
PUFAs	Polyunsaturated fatty acids
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SEM	Standard error of mean
TLR	Toll-like receptor
TRAP	Total peroxyl radical-trapping antioxidant parameter

Introduction

Besides nutritional effects, lipids also play structural and regulatory roles that have an important influence on the physiological functions of an organism, so they cannot be considered as only a source of energy. Polyunsaturated fatty acids (PUFAs) of ω-3 and ω-6 groups are considered to be the most powerful intracellular and intercellular mediators, precursors for the synthesis of eicosanoids, and modulators of the cell signalling network and cell membrane fluidity. This action of PUFAs can lead to various effects on immune and inflammatory processes [19, 24].

Phagocytes are a part of the innate immune system that plays a very important role in the initial response to infection. Their activation leads to the generation of the superoxide anion radical and the nitric oxide (NO) by NADPH oxidase and the inducible nitric oxide synthase (iNOS), respectively. Both the superoxide anion radical and the nitric oxide generate secondary reactive oxygen species (ROS) and reactive nitrogen species (RNS). Physiologically, ROS and RNS formation is one of the essential microbicidal mechanisms in an organism. Although the formation of reactive species is desirable for a host's defences, their overproduction can damage to the body's own cells, cause tissue injury, and contribute to the development of a number of serious diseases. Thus, the modulation of their production is an important target in the treatment of immune and inflammatory diseases [12, 20].

An oxidative burst of phagocytes such as neutrophils and macrophages accompanied by ROS and RNS production is one of the phagocyte functions that could be modulated by PUFAs [18, 21]. The effect of PUFAs on ROS and RNS formation depends on the carbon chain length and the number of double bonds, although one should note that ω-3 and ω-6 groups of PUFAs modulate their formation differently. However, studies focused on PUFAs modulation of ROS and RNS production are contradictory. PUFAs usually inhibit oxidative burst when added to diets, but they can either stimulate or inhibit ROS and RNS production when added directly to a cell culture [5, 14, 19, 20, 23]. Moreover, a high concentration of some PUFAs, or long and repeated exposure to their lower concentrations, can induce non-specific membrane damage; and, consequently, they can be toxic for many cell types [5, 7].

The aim of the present study was to evaluate and compare changes in ROS and RNS formation by stimulated macrophages treated with selected PUFAs representing both ω-3 (α-linolenic—ALA, docosahexaenoic—DHA, eicosapentaenoic—EPA) and ω-6 (arachidonic—AA, linoleic—LA) groups. Murine peritoneal macrophage line RAW 264.7 cultivated with PUFAs together with a known stimulator of immunologically active cells lipopolysaccharide (LPS) was used for our experiments. Luminol-enhanced chemiluminescence (CL) and spectrophotometrical detection of nitrites (the main and stable oxidative end product of NO chemistry) were used for the analysis of ROS and RNS production. Western blot analysis and scanning densitometry were used for the quantification of iNOS expression.

Materials and methods

Fatty acids tested

Fatty acids (arachidonic; α-linolenic; *cis*-4, 7, 10, 13, 16, 19-docosahexaenoic; *cis*-5, 8, 11, 14, 17-eicosapentaenoic; linoleic) were obtained from Sigma–Aldrich (Germany). Stock solutions of 0.1 M were prepared by dissolving fatty acids in 96% ethanol. Because PUFAs are very sensitive to oxidation, solutions were kept at −20 °C, under N_2 and in the dark. Immediately before use, PUFAs stock solution was diluted in a culture medium to reach the final concentrations of 0.1–1–10–100 μM. The final concentration of ethanol in the culture medium did not exceed 0.48% and was not toxic to cells.

Cell culture

Murine peritoneal macrophage cell line RAW 264.7 (American Type Culture Collection, USA) was cultivated in Dulbecco's Modified Eagle Medium (PAN, Germany) and supplemented with 10% of foetal bovine serum (PAN, Germany). Cells were maintained at 37 °C, 5% CO_2.

Detection of ROS production by chemiluminescence

Changes in ROS production were determined by a chemiluminescence (CL) method. Cells were incubated in

96-well plates at 1×10^5 cells/well for 8 h with LPS (0.1 μg/ml; Sigma, USA) and PUFAs at 37 °C, 5% CO_2. Then luminol (Molecular Probes, USA; final concentration 1 mM) and one of the NADPH oxidase activators—phorbol myristate acetate (PMA; Sigma, USA; final concentration 0.81 μM) or opsonized zymosan particles (OZP; Sigma, USA; final concentration 0.25 mg/ml)—were added. The controls were LPS-stimulated cells without PUFAs treatment and activated by PMA or OZP. Spontaneous CL (LPS stimulated, without activators) was also measured. CL signal was measured for 2 h at 37 °C. The integral representing the total amount of produced ROS was calculated for evaluation of the effects of PUFAs.

Measurement of nitrite concentration by Griess reaction

Changes in RNS production were measured indirectly as the accumulation of nitrites, the end product of NO metabolism, in a medium using spectrophotometrical Griess assay, as described previously [1]. Cells were incubated in 12-well plates at 1×10^6 cells/well for 20 h with LPS (0.1 μg/ml) and PUFAs at 37 °C, 5% CO_2. Control cells were incubated with LPS without PUFAs treatment. At the end of the incubation period, culture media were collected from wells and centrifuged at 5,000*g* and 4 °C for 5 min. Then, 150 μl of supernatant was mixed with an equal volume of Griess reagent (Sigma, USA) in a 96-well plate and the mixture was incubated at room temperature and in the dark for 30 min. The absorbance was measured at 546 nm. Sodium nitrite was used as the standard.

Western blot analysis of iNOS expression

After removing the supernatant for the nitrite measurement, the remaining cells were washed with cold phosphate buffered saline (PBS) and lysed in the lysis buffer (1% sodium dodecyl sulphate—SDS, 10^{-1} mol/l Tris pH 7.4, 10% glycerol, 10^{-3} mol/l sodium ortho-vanadate, 10^{-3} mol/l phenylmethanesulpfonyl fluoride). Protein concentrations were determined by using BCA™ protein assay (Pierce, USA), with bovine serum albumin as a standard. The equal amounts of protein were then subjected to SDS-polyacrylamide gel electrophoresis using 7.5% running gel. The expression of iNOS protein was quantified by Western blot analysis as described previously [3]. Anti-iNOS/NOS type II mouse monoclonal antibody (BIO-RAD, USA; 1:5,000) and ECL™ Anti-mouse IgG horseradish peroxidase linked whole antibody (from sheep; Biosciences, USA; 1:2,000) were used. The immunoreactive bands were detected using an ECL™ detection reagent kit (Pierce, USA) and exposed to radiographic film (AGFA, Belgium). Equal loading of proteins was confirmed by determination of β-actin. Relative protein levels were quantified by scanning densitometry using the Image J™ programme, and the individual band density value was expressed in arbitrary units.

ATP test of cell viability

Cell viability was determined luminometrically using the ATP Cell Viability test (BioThema, Sweden). Cells were incubated in 96-well plates at 1×10^5 cells/well for 8 or 20 h with LPS (0.1 μg/ml) and PUFAs at 37 °C, 5% CO_2. After incubation, the medium was removed from wells and Somatic cell ATP releasing reagent was added. After 15 min shaking, the ATP reagent was added, and the luminescence immediately measured. The intensity of CL was used for the evaluation of toxic effects of PUFAs tested on macrophages.

TRAP (total peroxyl radical-trapping antioxidant parameter) analysis

The luminol-enhanced CL assay for TRAP is based on the measurement of peroxyl radicals produced at a constant rate by a radical generator [17]. 2,2′-azobis (2-methylpropionamidine) dichloride (AMPA; Sigma, USA) was used as the peroxyl radical generator in our study. The TRAP value was determined from the duration of the period during which the CL signal was diminished by samples or standard antioxidant (for details see Ciz et al. [2]). Briefly, trolox, a water-soluble analogue of α-tocopherol, was used as a reference inhibitor. PBS, luminol and PUFAs sample or trolox were added to 96-well plates and incubated at 37 °C for 10 min. After incubation, AMPA was added, and the CL immediately measured.

Statistical analysis

The results are presented as the means from at least three independent experiments ± standard error of mean (SEM). Comparisons with control were performed by analysis of variance, followed by the Newman–Keuls post hoc test, or by the non-parametric Mann–Whitney test using Statistica for Windows 8.0 (Statsoft, USA). P values of <0.01 and 0.05 were considered significant.

Results

Toxicity of PUFAs

Fatty acids in all concentrations used had no significant effect on the viability or proliferation of RAW 264.7 cells, as measured by the ATP test (data not shown).

The effect of PUFAs on ROS production

The effect of PUFAs on ROS production by macrophages incubated for 8 h with LPS was analysed using luminol-enhanced CL. Typical kinetic curves of spontaneous macrophage CL and macrophage CL activated with receptor-bypassing (PMA) and receptor-operating (OZP) stimuli are shown in Fig. 1. Spontaneous CL was at the background level throughout the whole measurement period, which indicates that there was no increase in ROS production in non-activated cells treated with LPS. On the other hand, the activation of LPS-stimulated macrophages with both PMA and OZP induced a significant increase in ROS production. As demonstrated in Table 1, all PUFAs except EPA decreased ROS production in a 100 μM concentration. This effect was expressed most significantly when DHA was used. CL did not decrease as dramatically in the presence of AA; but, on the other hand, AA significantly ($P < 0.01$) decreased PMA-activated ROS production even in 1 and 10 μM concentrations. Interestingly, 10 and 100 μM EPA potentiated both PMA and OZP activated ROS production and OZP activated CL was potentiated even with 1 μM EPA.

The effect of PUFAs on nitrite production

To select the optimal incubation period for the analyses, nitrite production was detected up to 20 h after LPS stimulation. After a relatively short-term incubation period of up to 10 h, the concentration of nitrites was too low for the accurate detection of nitrite production (data not shown). The highest concentration of nitrites was observed after the 20-h treatment with PUFAs and LPS. Therefore, this incubation period was chosen for subsequent experiments. Table 1 demonstrates that all tested fatty acids in concentration 100 μM significantly ($P < 0.01$) decreased nitrite accumulation.

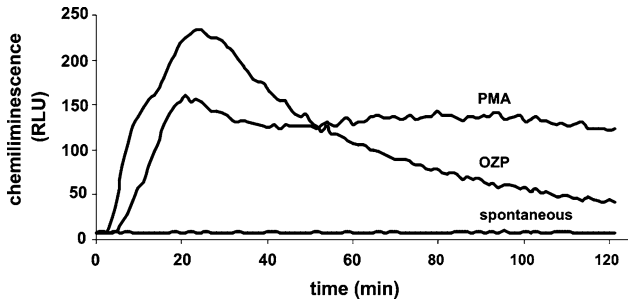

Fig. 1 Kinetics of luminol-enhanced activated and spontaneous CL of RAW 264.7 cells stimulated by LPS. The results are expressed in relative light units (RLU) and have been corrected for background

The effect of PUFAs on iNOS expression

The possibility that the changes in nitrite concentration were associated with iNOS protein expression was determined using Western blot analysis. In comparison with the iNOS protein level in the control sample, iNOS protein expression was partially inhibited by the highest concentration (100 μM) of ALA and significantly inhibited by the highest concentration of DHA (Fig. 2). DHA decreased the iNOS expression most effectively, as was similarly the case with nitrite accumulation in the cultured media.

Determination of antioxidant capacity of PUFAs

We analysed whether the decrease in phagocyte-derived CL can be caused by the antioxidant properties of PUFAs against ROS. The TRAP assay confirmed that none of the tested PUFAs exerts significant antioxidant properties against peroxyl radical (data not shown).

Discussion

Polyunsaturated fatty acids are well known to act as intracellular and intercellular mediators of immune response. The action of fatty acids depends on their chemical structure, carbon chain length, and number and position of double bonds. The effects of PUFAs can also differ depending on in vivo and in vitro conditions [14, 19]. Experimental and epidemiological observations indicate that beneficial properties of dietary lipids can be exploited in the treatment of immune and cardiovascular diseases, cancer, depression and many other serious diseases [6, 11, 13].

Modulation of ROS and RNS formation is one of the ways that PUFAs can affect the immune system [14]. ROS and RNS are produced by phagocytes such as neutrophils and macrophages after their stimulation with various types of molecules (bacterial LPS, other compounds of microorganisms, cytokines, etc.). Bacterial LPS is the routinely used stimulant of phagocytes. As an outer membrane component of bacteria, LPS can trigger the generation of a variety of inflammatory mediators and reactive oxygen and nitrogen intermediates by macrophages. Published studies demonstrate that 0.1 μg/ml LPS stimulates macrophages at a level that is sensitive to the anti-inflammatory effects of PUFAs [16]. Another reason for LPS application is the fact that no interaction between PUFAs and LPS or components of cell culture media was described.

Other authors have, in several in vitro studies, shown that the treatment of phagocyte cultures with PUFAs has a stimulating effect on ROS production. This action seems to

Table 1 Effect of tested PUFAs on nitrite production and PMA and OZP activated CL of LPS-stimulated RAW 264.7 cells

	PUFA	PUFAs concentration			
		0.1 [μM]	1 [μM]	10 [μM]	100 [μM]
Nitrites (% of control ± SEM)	AA	100.3 ± 1.1	100.7 ± 0.6	100.3 ± 0.9	87.7 ± 4.3**
	ALA	99.3 ± 0.1	99.2 ± 0.5	96.2 ± 1.0*	85.1 ± 1.5**
	DHA	98.9 ± 0.2	99.4 ± 0.1	97.7 ± 0.6	80.7 ± 2.7**
	EPA	99.7 ± 0.8	100.8 ± 1.2	98.6 ± 0.4	87.2 ± 1.3**
	LA	100.4 ± 0.7	101.3 ± 0.4	99.9 ± 0.4	92.0 ± 1.6**
PMA-activated CL (% of control ± SEM)	AA	95.6 ± 2.3	89.3 ± 1.9**	87.3 ± 2.2**	74.2 ± 2.6**
	ALA	94.9 ± 3.3	94.5 ± 4.2	91.2 ± 2.6	79.9 ± 4.5*
	DHA	104.5 ± 3.7	94.9 ± 2.9	92.0 ± 5.8	37.0 ± 7.2**
	EPA	96.4 ± 1.9	100.9 ± 2.3	112.8 ± 3.9*	112.9 ± 5.3
	LA	87.1 ± 5.3*	95.8 ± 0.4	100.5 ± 0.8	96.0 ± 1.7
OZP-activated CL (% of control ± SEM)	AA	94.4 ± 3.2	97.0 ± 2.8	100.2 ± 1.5	75.5 ± 1.7**
	ALA	98.7 ± 3.0	94.1 ± 0.6	89.8 ± 3.7	70.4 ± 3.2**
	DHA	100.2 ± 3.1	99.0 ± 5.3	92.09 ± 2.49	39.9 ± 9.3**
	EPA	97.8 ± 1.5	109.5 ± 1.9*	140.7 ± 1.3**	110.9 ± 5.8
	LA	95.9 ± 1.7	93.5 ± 4.3	97.4 ± 0.6	85.7 ± 2.6*

The data represent mean ± SEM from at least three independent experiments

The asterisks mark a significant difference (*$p < 0.05$, **$p < 0.01$) when compared with control value

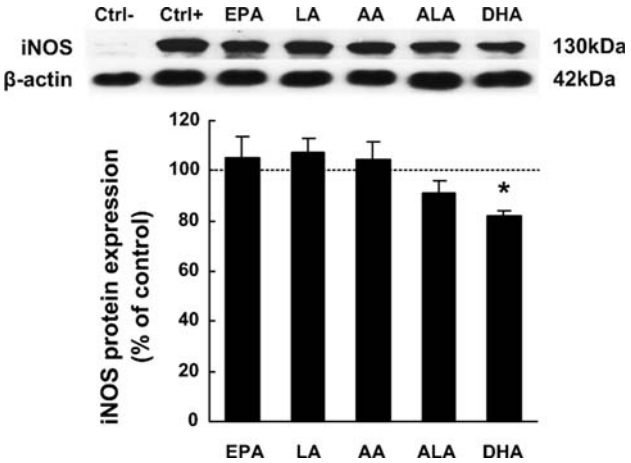

Fig. 2 Densitometric analysis and representative Western blot of iNOS protein expression in LPS-stimulated RAW 264.7 cells treated with 100 μM PUFAs. The data represent mean ± SEM from three independent experiments; the asterisks mark a significant difference (*$p < 0.05$) when compared with the control value (cells incubated only with LPS)

be less pronounced with increasing carbon chain length and an increasing number of double bonds: AA (20:4) > EPA (20:5) > DHA (22:6) [7, 9, 23]. On the other hand, Cockeran et al. [4] have demonstrated an inhibitory effect of DHA and EPA on ROS production measured luminometrically. Taken together, all these results show that the effects of PUFAs are very diverse, that these have not been precisely described, and that further investigation is needed.

Therefore, in our study, we tested the hypothesis that the modulation of ROS formation is one of the ways that PUFAs can affect macrophages. Using luminol-enhanced chemiluminescence, we were able to detect both intracellular and extracellular production of ROS [8]. Our experiments showed that, with the exception of EPA, the PUFAs tested did not increase the production of ROS by LPS-stimulated RAW 264.7 cells. The highest concentrations (100 μM) of AA, ALA, DHA and LA decreased the formation of ROS. In this concentration DHA had the highest inhibitory effect of all the acids tested in both PMA and OZP-activated chemiluminescence. AA inhibited PMA-activated ROS production even in 1 and 10 μM concentrations, but no marked difference was observed in OZP-activated ROS production. Interestingly, EPA in concentrations higher than 1 μM stimulated both PMA and OZP-activated ROS production.

It is difficult to reveal the mechanism responsible for the observed effects of PUFAs on ROS production in LPS-stimulated macrophages, especially for whole cells where PUFAs can act either directly (NADPH oxidase) or indirectly via the signal transduction system, modulating cell membrane fluidity or eicosanoide production [4, 14]. since it is evident from our results, that, due to the absence of antioxidative properties, none of the tested PUFAs scavenged ROS, we suppose that their inhibitory action cannot be attributed to this phenomenon. Focusing on the effect of PUFAs on ROS production in neutrophils, it is known that AA and LA liberated by phospholipase A_2 from membrane

phospholipids are essential for NADPH oxidase activation, since they can interact directly with p45phox or p67phox subunits by unmasking their membrane binding domains or can interact with other relevant regulatory components [22, 23]. Our results imply that only EPA which dose dependently increased ROS production in LPS-stimulated macrophages can affect NADPH oxidase in a similar way. Regarding the effect of other PUFAs, we assume that, in contrast to neutrophils, AA, LA together with ALA and DHA can negatively affect the activation of NADPH oxidase in LPS-stimulated macrophages. Our observations, together with results from other studies [14, 19], indicate a specific mechanism of PUFAs action in LPS-stimulated macrophages and their role in the regulation of ROS production, which remains to be elucidated.

A further important finding of this paper is that all of the PUFAs tested markedly inhibited NO production by LPS-stimulated macrophages. One of the possible explanations for the inhibitory action of PUFAs could be the scavenging activity against NO. Nevertheless, none of the PUFAs tested was able to scavenge NO, as we tested by electrochemical measurements (unpublished results). Therefore, we conclude that the decrease in NO production without the influence on iNOS protein expression in LPS-stimulated cells has to be caused by another mechanism. Our results imply that AA, LA, ALA and EPA, which reduced nitrite accumulation without decrease in iNOS protein expression, are able to modulate NO production in LPS-stimulated macrophages through a mechanism that seems to be independent of the regulation of iNOS protein expression. DHA was the only fatty acid that was able to significantly reduce the iNOS protein expression and can probably interfere with the intracellular signalling pathways associated with iNOS regulation. It has previously been demonstrated that PUFAs could inhibit the LPS-induced activation of the nuclear transcription factor NFκB [10] and other inflammatory markers in the RAW 264.7 cell line by modulating the activation of TLR-4 (Toll-like receptor-4) and, thus, prevent activation of several genes, including those for iNOS [14, 15].

On the other hand, De Lima [5] described stimulation of NO production induced by low concentrations of PUFAs and decreased NO production at high doses of PUFAs (50–200 μM) after 48 h treatment. These authors explain this inhibitory effect by the toxic effect of PUFAs in these concentrations on the J774 cell line. However, we did not observe the cytotoxic effects of PUFAs in any of the tested concentrations (up to 100 μM). This difference may be due to the better tolerance of RAW 264.7 cells to high concentrations of PUFAs or shorter incubation periods.

In conclusion, PUFAs are able to affect ROS and RNS formation by LPS-stimulated macrophages. The observed effects, together with the absence of antioxidant activity and cytotoxic properties, indicate that PUFAs can participate in the regulation of the enzymes responsible for the reactive species production. We did not observe a connection between the structure of the chain of PUFAs and their effects on reactive species formation. DHA, ω-3 PUFA with the longest carbon chain and the highest number of double bonds from PUFAs tested, showed the strongest inhibitory effect on both ROS and RNS formation. EPA also significantly inhibited RNS production. However, despite the similarities in hydrocarbon chain structure of these two ω-3 PUFAs, ROS production was potentiated by EPA. We suppose that anti-inflammatory actions of DHA could contribute to the prevention and treatment of some immune and inflammatory diseases.

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