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BIOTRANSFORMATION OF ORGANIC NITRATES TO NITRIC OXIDE BY VASCULAR SMOOTH MUSCLE AND ENDOTHELIAL CELLS

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Summary: The vasodilator action of organic nitrates is thought to be mediated by an increase in the level of cGMP following stimulation of the cytosolic enzyme guanylate cyclase in the vascular smooth muscle cell. However, direct evidence for the formation of the putative active metabolite, nitric oxide (NO) within the different compartments of the vascular wall is still missing. We here demonstrate for the first time that cultured vascular smooth muscle cells as well as endothelial cells from different species actively metabolize organic nitrates to NO. We furthermore present evidence for an outward transport of cGMP from both cell types following stimulation of soluble guanylate cyclase. The rate of NO release closely correlated with the rate of cGMP egression. Biotransformation of organic nitrates to NO appeared to comprise at least two different components, a heat-sensitive enzymatic pathway which is short-lived and prone to rapid desensitization and a second non-enzymatic component which is apparently unsaturable and longer lasting. The marked decrease in the release of NO and cGMP upon the repeated administration of organic nitrates suggests that the phenomenon of "nitrate tolerance" is mainly due to an impaired biotransformation. We propose that the metabolism of nitrates to NO may have important implications for the prevention of atherosclerosis and the therapeutic modulation of blood cell function.

Despite the beneficial therapeutic use of organic nitrates such as GTN in coronary heart disease for more than a century their mode of action at the cellular level remained obscure until recently[1]. We know today that these compounds are prodrugs in that they release NO which increases intracellular levels of cGMP via stimulation of the soluble isoenzyme of guanylate cyclase. Organic nitrates thus mimic the action of endothelium-derived relaxing factor, which is an important endogenous modulator of vascular tone[2]. Brien et al. have recently shown that both biotransformation of GTN to the respective dinitrates and enhancement of intracellular cGMP levels precedes the onset of vascular relaxation, confirming that metabolic activation of organic nitrates is a prerequisite for their pharmacodynamic action[3]. A

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Abbreviations:
NO: nitric oxide; cGMP: cyclic guanosine monophosphate; GTN: glyceryl trinitrate; ISDN: isosorbide dinitrate; HbO_2: oxyhaemoglobin; MetHb: methaemoglobin.

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close correlation was found between the rate of NO formation from and the extent of guanylate cyclase stimulation, by organic nitrates in vitro[4]. A study demonstrating the metabolism of organic nitrates to NO by the different cell types of the vascular wall and their contribution to the intracellular events leading to increased levels of cGMP and vessel relaxation, however, is still lacking. We therefore developed a technique which allows continuous monitoring of the release of NO and cGMP from vascular cells cultured on microcarrier beads by means of difference-spectrophotometry and radioimmunoassay. We here demonstrate that vascular smooth muscle cells metabolize organic nitrates to NO and that the capacity for this biotransformation decreases rapidly upon continuous or repeated nitrate application. In addition, endothelial cells, which have been regarded previously as an inert cell layer in regard to nitrate metabolism, convert organic nitrates to NO as efficient as do smooth muscle cells. The role of the endothelium in modulating vascular responses to organic nitrates therefore requires careful reevaluation. We furthermore present evidence that an intracellular formation of NO results in the egression of cGMP from the generating cell. The diminished rate of biotransformation to NO upon the continuous or repeated application of GTN implies an important role of this process in the development of nitrate tolerance.

MATERIALS AND METHODS

Cell Culture:
Endothelial cells were harvested from porcine thoracic aorta with collagenase, cultured in M199 medium with 20% foetal calf serum and characterized by their typical contact-inhibited "cobblestone-like" growth pattern and the uptake of acetylated low density lipoproteins. Absence of smooth muscle cell or fibroblast contamination was verified by counterstaining for α-actin. Endothelial cells of passage 1 were grown to confluence on microcarrier beads (Biosilon, Nunc). 1 ml of endothelium covered beads corresponded to 2.3×10^7 cells and 4.8 mg protein, respectively. Vascular smooth muscle cells were isolated by explant technique from fresh macrovascular aortic tissue of different animal species and cultured in M199 with 10% foetal calf serum. The smooth muscle cells revealed the typical "hill and valley" growth pattern when assessed by phase-contrast microscopy and were stained with a specific antibody against smooth muscle α-actin. Cells were seeded on microcarrier beads 2-3 weeks after isolation. 1 ml of smooth muscle cell covered beads corresponded to 14-18 mg protein.

Measurement of NO and cGMP:
Endothelial or vascular smooth muscle cells on beads were packed into a water-jacketed chromatography column and perfused at 2.0 ml/min with a modified Krebs-Henseleit buffer (pH 7.40, 37°C, pO_2 150-160 mm Hg) supplemented with 5 μM freshly prepared HbO_2. The volume of cell-covered beads varied between 3 and 3.5 ml. The column effluent was continuously passed through the flow-cell of a dual wavelength spectrophotometer (Shimadzu UV-3000). Formation of NO was continuously monitored by a specific difference-spectrophotometric technique which is based on the NO-induced conversion of HbO_2 to MetHb[4,5]. Since the presence of HbO_2 in the perfusion buffer ensures the effective trapping of extracellularly released NO, the formation of MetHb in the cellular effluent is a direct measure for the generation of NO. Calculation of NO concentrations was based on a molar extinction coefficient of 38.7 $mM^{-1}cm^{-1}$ (401.5 vs 411 nm) and was verified by infusion of standard solutions of authentic NO. Detection limit of the assay (signal to noise ratio > 3:1) was determined to be 1.5×10^{-11} moles NO/min at the given flow rate of 2 ml/min. Nitrate compounds

and bradykinin were applied as constant infusion (5-45 μl/min) over the cells by means of high precision infusion pumps. For the measurement of released cGMP aliquots of the column effluent were collected at 90 s intervals, immediately put on ice and subsequently evaporated to dryness. The concentration of cGMP in these fractions was determined by means of radioimmunoassay. Cell integrity before and after the experiments was checked by scanning electron microscopy and measurement of lactate dehydrogenase release. Reported rates for NO formation and cGMP egression were expressed as increases from baseline values.

Materials:
GTN and ISDN were diluted from commercial aqueous formulations (perlinganit^{R} and Isoket^{R}, Schwarz Pharma AG, Monheim, Germany) in saline. Buffer salts were of analytical grade and purchased from Merck, Darmstadt, all other compounds were of the highest purity available and purchased from Sigma, Taufkirchen, Germany.

RESULTS AND DISCUSSION

There was no detectable formation of NO from cultured porcine vascular smooth muscle cells under basal conditions. Short-term infusion of GTN over the cells (1-400 μM, 1-3 min) resulted in an immediate, concentration-dependent formation of NO. The time delay for the onset of NO release was typically less than 1 s, indicating rapid cellular uptake and metabolism of the nitrate. The formation of NO was biphasic and peaked at 53 ± 5 s after the onset of the reaction with a progressive decline to 36 ± 7 % of the maximal rate at 3 min (n=6). After cessation of the GTN infusion NO formation returned to baseline levels within 90 ± 12 s at concentrations up to 100 μM. Superfusion of the cells with higher concentrations of GTN resulted in significantly prolonged formation of NO, pointing to an intracellular accumulation of the nitrate. A coinfusion of SOD (100 U/ml) had no influence on the apparent rate of NO formation from GTN (n=2, data not shown). Peak formation of NO from 100 μM GTN amounted to 78 ± 14 pmoles/min x ml bead volume. The net conversion to NO during a 3 min infusion with 100 μM GTN was calculated to be 0.087 ± 0.018 %, taking into account the absolute amounts of GTN applied and NO formed. Although both the maximal rate of formation and the absolute amount of NO increased with rising concentration of GTN, the total turn-over progressively decreased, indicating saturation of the metabolizing system. The conversion rate to NO fell from 0.15 % at 5 μM to 0.09 % at 100 μM GTN. Similar results were obtained with ISDN, although NO generation rates were approx. 10-fold lower than those observed with GTN (data not shown); this is in agreement with a weaker potency of this compound in isolated organ preparations.

When GTN was applied as long-term infusion over the cells (100 μM for 30 min) there was a rapid decline in the formation of NO within the first 10 min to 32 ± 9 % of the maximal response with minimal further decrease. When the same concentration of GTN was applied 30 min afterwards, maximal NO formation was comparable to that at the end of the 30 min stimulus. Repeated 3 min infusions of 100 μM GTN resulted in a progressive decrease in NO formation from 78 ± 14 to 9 ± 2 pmoles NO/min x ml bead volume from the first to the fifth stimulus (Fig.1). The total extent of conversion of GTN to NO was reduced from 0.087 % to 0.015 % under the same conditions.

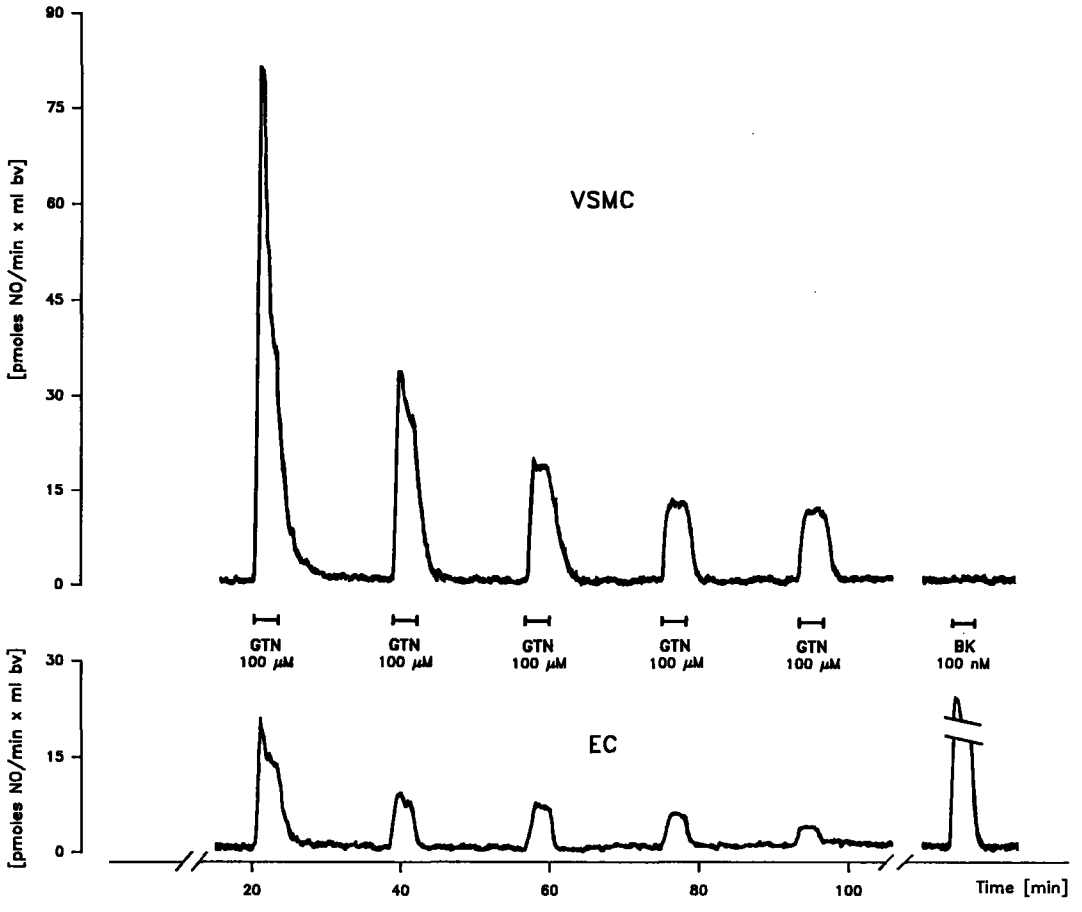

Fig.1. Biotransformation of GTN to NO by cultured vascular smooth muscle cells (VSMC) and endothelial cells (EC). GTN was repeatedly applied at 100 µM for 3 min with drug-free intervals of 15 min. Representative tracings (compensated for baseline drift) of 6-8 separate experiments with each cell type. For a quantitative comparison of NO formation the different protein content per bead volume (bv) must be taken into consideration (see Methods).

To evaluate whether the observed process of NO generation from organic nitrates was species-specific, cultured smooth muscle cells from guinea pig and rat aorta were additionally investigated. Smooth muscle cells from both species metabolized organic nitrates to NO with approx. 2 and 20 fold weaker efficiency than the corresponding porcine cells, demonstrating that this biotransformation pathway does occur but with different efficacies in vascular smooth muscle cells of several species.

Although it is well documented that the endothelium is capable of generating NO upon stimulation with endogenous mediators such as bradykinin (see Fig.1), nothing is known about its ability to convert prodrugs such as organic nitrates to NO. Basal release of NO from porcine aortic endothelial cells in this study ranged from 2.3 to 3.1 pmoles NO/min x mg protein. Superfusion of the cells with GTN resulted in the formation of NO with a profile similar to that observed with smooth muscle cells (Fig.1). Calculations based on protein content revealed that endothelial cells metabo-

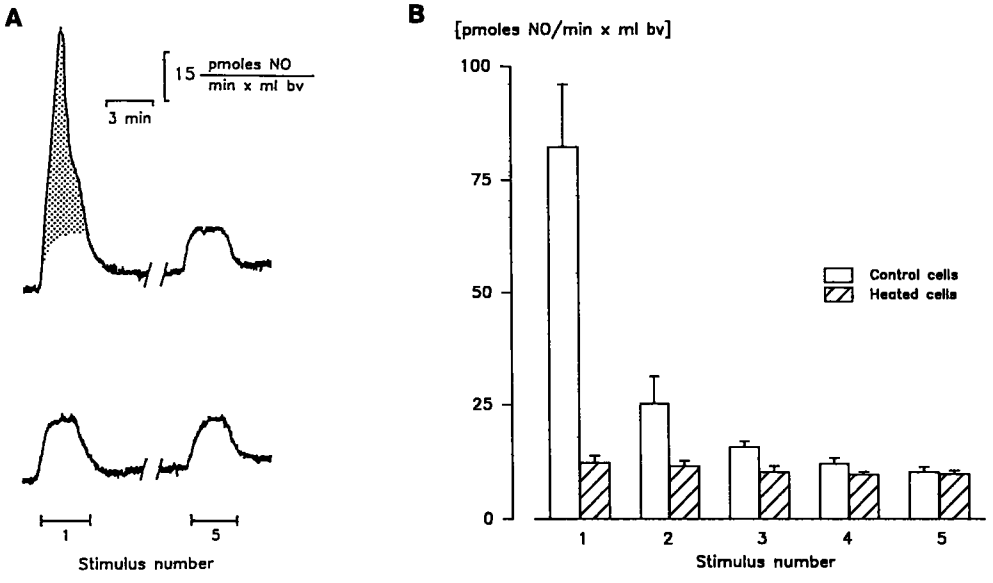

Fig.2. A. Discrimination of NO formation pathways by cell heating. Identical batches of porcine aortic smooth muscle cells on microcarrier beads were filled in two separate columns. One cell portion (lower panel) was subjected to heating at 56°C for 90 min prior to challenge with 100 μM GTN, whereas the other portion (upper panel) served as a control. The hatched portion of the peak from the untreated cells reflects the heat-sensitive component of GTN metabolism. Representative tracing from 3 separate experiments. **B. Decrease in the rate of NO formation upon the repeated application of GTN. Comparison of native and thermally treated smooth muscle cells.** Maximal NO formation rates in response to the repeated superfusions of smooth muscle cells with 100 μM GTN for 3 min were calculated from peak NO concentrations taking flow rate and bead volume into account. Reported data are mean values from 3 experiments which were normalized to 1 ml packed bead volume (bv) and corrected for readings obtained under the same conditions without cells.

lized GTN as effectively as did vascular smooth muscle cells (data not shown). Results from primary cultures of endothelial cells grown on microcarrier beads 3 days after isolation were not significantly different from those obtained with cells of passage 1 (n=2). Endothelial cells did not appear to have any inhibitory or stimulatory effect on GTN metabolism in vascular smooth muscle cells as NO formation rates from endothelium/smooth muscle cocultures (initial cell ratio at the time of seeding on beads 50:50) were between those of the single cell types (n=2, data not shown).

In experiments designed to elucidate the nature of the cellular transformation of organic nitrates to NO, porcine aortic smooth muscle cells on microcarrier beads were subjected to heating at 56°C for 90 min prior to measurement. The profile of NO formation from these pretreated cells differed markedly from those of control cells in that maximal formation rates were significantly lower and no longer biphasic (Fig.2). The absence of the short-lived component of GTN metabolism after heating points to a temperature-sensitive enzymatic pathway involved in organic nitrate biotransformation. When control cells were heated between the fifth and the sixth stimulus no further decrease in NO formation was observed (Fig.3), pointing to the presence of a second component of GTN metabolism with apparently different characteristics. The

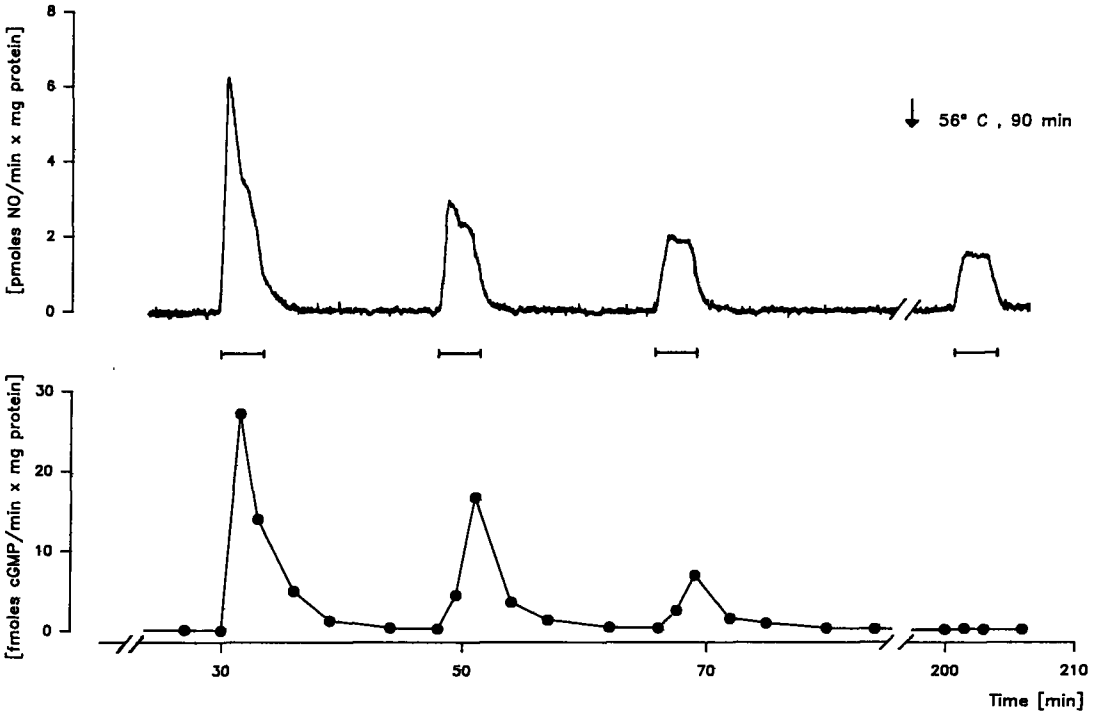

Fig.3. Comparison of NO formation and cGMP egression from porcine aortic smooth muscle cells upon repeated stimulation with 100 μM GTN. Loss of cGMP release after cell heating. Representative tracing from 2 separate experiments.

decrease in NO formation upon the repeated application of GTN was mainly attributable to desensitization of the heat-sensitive component (Fig. 2B), which is probably of enzymatic nature. The second component of nitrate biotransformation may either be mediated by a heat-stable enzyme system or by a non-enzymatic pathway of NO formation.

As increasing drug-free intervals from 10 to 120 min failed to restore maximal formation of NO, experiments were performed to test whether the apparent desensitization of the heat-sensitive component of GTN metabolism is due to the consumption of intracellular thiols. Attempts were undertaken to replenish intracellular thiol-stores by infusing 500 μM of either N-acetyl-DL-cysteine or reduced L-glutathione for 30 min between the fifth and the sixth nitrate stimulus. Both thiols as well as NADPH (100 μM, 15 min) failed to restore the initial formation of NO from GTN. Moreover, a coinfusion of N-acetylcysteine starting 15 min before the first GTN stimulus did not prevent desensitization of the metabolising system.

Release of cGMP from cultured vascular smooth muscle cells under basal conditions was below the detection limit of the assay (< 20 fmoles cGMP/min x ml bead volume). Superfusion of the cells with organic nitrates induced the egression of cGMP in a concentration-dependent manner. ISDN induced an approx. 10-fold lower

release of cGMP than GTN (data not shown). Maximal cGMP egression decreased from the first to the fifth stimulus of GTN (Fig. 3). Smooth muscle cells which had been subjected to heating at 56°C for 90 min failed to release cGMP in response to organic nitrates despite a still measurable formation of NO, which may be due to a thermally-induced alteration of guanylate cyclase, an impairment of the outward transport for cGMP, or both. Organic nitrates also induced the egression of cGMP from cultured endothelial cells with a gradual decrease upon repeated application. In all experiments the pattern of cGMP egression from cultured vascular cells closely matched that of NO formation ($r=0.96$, $n=12$), confirming its role as second messenger of the action of NO-releasing drugs. To our knowledge this is the first demonstration of an egression of cGMP from cultured vascular cells following stimulation of soluble guanylate cyclase which may, at least in part, explain the discrepancy between measured intracellular levels of cGMP and the extent of vasodilatation in response to the application of nitrovasodilators[6]. In addition to cyclic nucleotide phosphodiesterase activity the egression of cGMP appears to be an effective mechanism for the reduction of increased intracellular nucleotide levels. Whether this is merely passive or actively controlled by any transport system remains to be investigated. It is intriguing that although both basal and stimulated levels of cGMP are reported to be rather low in endothelial cells compared to smooth muscle cells[7,8], in the present study the rate of NO and cGMP release upon stimulation with organic nitrates was of the same order of magnitude in both cell types of porcine origin. This points to an effective outward transport for cGMP in endothelial cells, which may be involved in a cGMP-regulated feedback system allowing low steady-state levels of cGMP in cells that under basal conditions already have a high formation of NO.

In conclusion, the present study shows that organic nitrates are metabolized to NO with comparable efficacy in endothelial and vascular smooth muscle cells. This biotransformation appears to comprise at least two distinct pathways, the nature and contribution of which to the pharmacodynamic action of organic nitrates remains to be investigated. In both cell types the conversion of organic nitrates to NO and the subsequent formation of cGMP are highly susceptible to desensitization. These data extend previously published results which suggest that the phenomenon of "nitrate tolerance" is the result of an impaired biotransformation rather than the expression of neurohormonal counterregulation or an alteration of the target enzyme guanylate cyclase[9,10]. The present experimental approach will allow to systematically test the effect of specific enzyme inducers and inhibitors in order to elucidate the subcellular nature of organic nitrate metabolism opening up the possibility for a rational drug design suited to overcome the problem of nitrate tolerance.

An important advantage of organic nitrates over compounds which enhance the release of endogenous NO is that even at the sites of endothelial injury, NO arising from the metabolism of nitrates will effectively inhibit the adhesion of blood cells such as leukocytes, platelets and macrophages to the vascular wall. Together

with the recent finding that nitrates are able to inhibit the proliferation of smooth muscle cells[11], this may have important implications for the prevention of atherosclerosis, in which the crucial pathogenic steps include the attachment of leukocytes to the vascular wall, their migration through the intimal layer and, as a result, an increased rate of myocyte proliferation in the subendothelial cell layer.

Since the release of nitrate-derived NO from endothelial cells is not only directed to the musculature, causing relaxation and inhibition of cell proliferation, but also to the luminal site, thereby inhibiting blood cell adhesion and aggregation, we propose that this biotransformation pathway may moreover have important implications for the modulation of blood cell function. It may thus be the long-sought key for the understanding of why organic nitrates inhibit platelet aggregation in vivo whereas they generally display only poor effectiveness under in vitro conditions and is likely to be of major therapeutic importance in pathophysiological disorders which are associated with an enhanced platelet aggregability. Inhibition of neutrophil accumulation by the formation of NO from organic nitrates may furthermore play a beneficial role in preserving endothelial integrity and limiting reperfusion injury following myocardial infarction.

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