Renal sympathetic nerve activity during asphyxia in fetal sheep

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Submitted 9 February 2012; accepted in final form 26 April 2012

Booth LC, Malpas SC, Barrett CJ, Guild S, Gunn AJ, Bennet L. Renal sympathetic nerve activity during asphyxia in fetal sheep. Am J Physiol Regul Integr Comp Physiol 303: R30–R38, 2012. First published May 2, 2012; doi:10.1152/ajpregu.00063.2012.—The sympathetic nervous system (SNS) is an important mediator of fetal adaptation to life-threatening in utero challenges, such as asphyxia. Although the SNS is active well before term, SNS responses mature significantly over the last third of gestation, and its functional contribution to adaptation to asphyxia over this critical period of life remains unclear. Therefore, we examined the hypotheses that increased renal sympathetic nerve activity (RSNA) is the primary mediator of decreased renal vascular conductance (RVC) during complete umbilical cord occlusion in preterm fetal sheep (101 ± 1 days; term 147 days) and that near-term fetuses (119 ± 0 days) would have a more rapid initial vasomotor response, with a greater increase in RSNA. Causality of the relationship of RSNA and RVC was investigated using surgical (preterm) and chemical (near-term) denervation. All fetal sheep showed a significant increase in RSNA with occlusion, which was more sustained but not significantly greater near-term. The initial fall in RVC was more rapid in near-term than preterm fetal sheep and preceded the large increase in RSNA. These data suggest that although RSNA can increase as early as 0.7 gestation, it is not the primary determinant of RVC. This finding was supported by denervation studies. Interestingly, chemical denervation in near-term fetal sheep was associated with an initial fall in blood pressure, suggesting that by 0.8 gestation sympathetic innervation of nonrenal vascular beds is critical to maintain arterial blood pressure during the rapid initial adaptation to asphyxia.

METHODS

Experimental preparation. All procedures were approved by the Animal Ethics Committee of The University of Auckland. Four groups of time-mated singleton Romney/Suffolk fetal sheep were instrumented at the following ages: preterm-occlusion (PT) at 99 ± 1 days gestation (n = 7, term = 147 days), near-term-occlusion (NT) at 119 ± 0 days gestation (n = 6), preterm unilateral renal denervation (PT-denervation) at 99 ± 0 days gestation (n = 6), and sympathectomized near-term fetal sheep (NT-6-OHDA) at 121 ± 1 days gestation (n = 3).

Food, but not water, was withdrawn 18 h before surgery. Ewes were given 5 ml of Streptocin [procaine penicillin (250,000 IU/ml) and dihydrostreptomycin (250 mg/ml, Stockguard Labs, Hamilton, New Zealand)] intramuscularly for prophylaxis 30 min prior to the start of surgery. Anesthesia was induced by intravenous injection of Alfaxan (Alphaxalone, 3 mg/kg; Jurox, Rutherford, NSW, Australia), and general anesthesia was maintained using 2–3% isoflurane in O2.
The depth of anesthesia, maternal heart rate, and respiration were constantly monitored by trained anesthetic staff. Under anesthesia, a 20-gauge intravenous catheter was placed in a maternal front leg vein, and the ewes were placed on a constant infusion isotonic saline drip (at an infusion rate of ~250 ml/h) to maintain maternal fluid balance. Briefly, fetal hindlimbs and abdomen were exposed through a midline cesarean incision, and a small incision in the uterus (13, 14, 71). The left femoral artery was isolated and catheterized to measure mean arterial blood pressure (BP). In the PT and NT groups, the right kidney was exposed via a retroperitoneal incision and a 2SB-type ultrasonic blood flow probe (Transonic Systems, Ithaca, NY) was placed around the renal artery to measure renal blood flow (RBF), and the left kidney was exposed via a second retroperitoneal incision, the renal sympathetic nerve was visualized with a surgical microscope (OPMI 1FC, Zeiss, Oberkochen, Germany), and the electrode coils of a telemetry-based implantable nerve amplifier (Telemetry Research Limited, www.telemetryresearch.com, Auckland, New Zealand) were coiled around the nerve. The electrode and nerve were insulated from the surrounding tissues with a coat of silicone elastomer (Kwik-sil, World Precision Instruments, Sarasota, FL) (6). The implantable amplifier was then secured on to the back of the fetus. To ensure that continuous signals were recorded, an aerial was secured on to the back of the fetus (13). In the PT-denervated group, only one kidney was exposed, and the renal sympathetic nerves were visualized and cut. The surrounding area and outer surface of the renal artery were then “painted” with 70% ethanol to destroy smaller nerves. A 2SB-type ultrasonic blood flow probe was fitted around the artery.

The uterus was then closed in layers, and a second incision was made to expose the fetal head and upper chest. Polyvinyl catheters were placed in the fetal right brachial artery for withdrawal of preductal arterial blood samples and the amniotic sac. Electrocardiogram electrodes (Cooner Wire, Chatsworth, CA) were sewn across the fetal chest to record the fetal heart rate (HR). A Teflon-coated stainless-steel electrode (Cooner Wire ) was sewn in the nuchal muscle to record electromyographic activity (EMG) as a measure of fetal movement, and a reference electrode was sewn over the occiput. A 2SB-type ultrasonic blood flow probe was fitted around the renal artery to measure renal blood flow (RBF), and the left kidney was exposed using the timing of the initial rapid fall in RVC after occlusion (13, 50).

Confirmation of renal denervation in preterm fetal sheep. Twelve fetal kidney samples were analyzed for norepinephrine content to confirm renal denervation using HPLC [A/Prof Tim Yandle, Endolab, Christchurch Cardioendocrine Research Group, New Zealand (57)]. Briefly, intact and denervated kidneys were removed, frozen in liquid nitrogen, and stored at −80°C. Kidney samples (0.5 g) were homogenized with 5 ml of 0.4 M perchloric acid (with 5 mM reduced glutathione) and 100 μl of 3.5 μM dihydroxybenzylamine and centrifuged at 4,500 rpm at 4°C for 15 min. The supernatant was used to calculate the catecholamine concentrations. Denervated kidneys had an average norepinephrine content of 7,341 ± 550 pg/g compared with an average of 16,507 ± 6,325 pg/g in intact kidneys (n = 6; P < 0.05).

Experimental design. Experiments were conducted at 101 ± 1 (PT), 121 ± 0 (NT), 104 ± 0 (PT-denervated), and 128 ± 1 (NT-6OHDA) days gestation, between 24 and 72 h after surgery. Fetal BP (corrected by subtraction of intra-aminotic pressure), HR, EMG, RBF, and RSNA (control groups only) were recorded and saved continuously to disk for off-line analysis using custom data acquisition programs (LabView for Windows; National Instruments, Austin, TX). Data were recorded for 12 h prior to the experiment.

Asphyxia was induced by rapid inflation of the umbilical cord occluder with a predetermined volume of sterile saline for 12 min in near-term fetal sheep and 20 min in preterm fetal sheep. Complete occlusion was confirmed by bradycardia and hypertension; all fetuses completed the full period of occlusion. Arterial blood samples were taken 30 min before the start of occlusion and during (10 min in NT group, 12 min in NT-6OHDA group, and 17 min in both PT groups) occlusion for preductal pH, blood gas (845 blood gas analyzer and cooximeter; Ciba-Corning Diagnostics, MA), glucose, and lactate measurements (model 2300; Yellow Springs Instruments, Yellow Springs, OH). At the end of the protocol, the ewe and fetus were killed with an overdose of pentobarbital sodium (9 g iv to the ewe; Pentobarb 300, Chemstock International, Christchurch, New Zealand).

Data analysis. RSNA signals were amplified 50,000 times, filtered between 50 and 2,000 Hz, full-wave rectified, and integrated using a low-pass filter with a time constant of 20 ms (6, 13). The analog signals were then digitized and continuously displayed and recorded at 500 Hz. For 5-s analysis, nerve activity was expressed as a percentage of baseline (baseline; 15 min prior to occlusion). Confirmation of the RSNA signal was established by the presence of coordination between the bursting pattern in sympathetic activity and the cardiac cycle, on averages of 1-s intervals of blood pressure and RSNA obtained using the systolic pressure as a trigger, for 200 epochs (13, 50).

The detection limit of the probes used to measure RBF was 0.01 ml/min. RVC was calculated using the formula: blood flow (ml/min)/BP (mmHg) (9). Conductance was calculated rather than the reciprocal, vascular resistance, because during asphyxia, peripheral blood flow falls to near zero, leading to highly nonlinear numerical changes.

Statistics. Statistical analysis was performed using SPSS (SPSS, Chicago, IL). Changes in cardiovascular variables were analyzed for key stages of asphyxia separately (0–1 min and 2–6 min) using repeated-measures ANOVA; where appropriate, the Huynh-Feldt correction was applied. Changes in key features of cardiovascular changes (particularly, the maxima or nadir of changes) and the percent of maximal change in RSNA and RVC over the first 50 s (corresponding with the timing of the initial rapid fall in RVC after occlusion) were tested by repeated-measures ANOVA. Individual amplitudes and times were calculated and reported as means ± SE. Statistical significance was accepted when P < 0.05.

RESULTS

Biochemistry measurements during occlusion. Before umbilical cord occlusion, all fetuses (NT, n = 6; PT, n = 7; NT 6OHDA, n = 3, PT-denervation, n = 6) had normal blood gas, pH, and glucose-lactate status for their respective gestations.
according to our laboratory standards (71). Umbilical cord occlusion was associated with severe hypoxia, hypercapnia, and mixed metabolic and respiratory acidosis (Table 1).

**HR, BP, RBF, RVC, and RSNA during umbilical cord occlusion.** Umbilical cord occlusion was associated with a brisk fall in HR in both NT and PT fetuses (Fig. 1). HR was higher in PT fetuses than NT \( (P < 0.005) \) in the first minute of occlusion. There was a marked increase in BP that was greater in NT compared with PT fetal sheep, both in peak and at 1 and 2–6 min occlusion \( (P < 0.005) \). RBF fell significantly in both NT and PT fetuses, to a lower nadir in NT than PT fetuses \( (P < 0.005) \). RBF (%baseline) fell significantly more in NT than PT fetal sheep \( (P < 0.05) \). The pattern of changes in RVC was similar, with a more rapid initial fall in NT than in PT fetuses \( (73 \pm 8 \ vs. \ 20 \pm 6\% \ from \ 0 \ to \ 50 \ s, \ P = 0.02) \) to a significantly lower nadir \( (P < 0.05) \).

RSNA increased after occlusion in both NT and PT fetuses, with an increase in both the number and size of the bursts of RSNA (Fig. 2) at a time when body movements, as measured by nuchal electromyographic activity, were suppressed. The initial increase in RSNA was markedly less than the change in RVC \( (20 \pm 6 \% \ and \ 6 \pm 6 \% \ of \ the \ maximal \ increase \ occurred \ 0–50 \ s \ in \ NT \ and \ PT \ fetuses, \ respectively, \ P < 0.001 \ vs. \ RVC) \). The maximum increase in RSNA tended to be greater in NT than PT fetuses \( (P = 0.07) \), and peak RSNA was reached significantly later \( (P < 0.05) \). The sharp fall in HR in the first minute of asphyxia was associated with periods of atrioventricular block (Fig. 3). During these periods, there were multiple bursts of RSNA for each cardiac cycle.

**Unilateral denervation in preterm fetal sheep.** Unilateral denervation in PT fetal sheep was not associated with any significant differences compared with intact PT fetuses (Fig. 4).

**Chemical sympathectomy in near-term fetal sheep.** Chemical sympathectomy with 6-OHDA was associated with an initial transient fall in BP maximal after 35 s \( (P < 0.05 \ vs. \ intact \ NT \ fetuses, \ Fig. 5) \) and a parallel exaggeration of the initial fall in RBF \( (P < 0.05) \). RVC was not significantly different from intact animals in the first minute of asphyxia, although there was an apparent trend for RBF and RVC to be higher in 6-OHDA-treated animals compared with controls between 2 and 6 min of occlusion \( (P < 0.066) \).

**DISCUSSION**

The present study demonstrates for the first time that fetal RSNA increases substantially in 0.7 gestation preterm fetal sheep during profound, life-threatening asphyxia induced by umbilical cord occlusion. With increased maturation, there was a more sustained increase in RSNA, with RSNA reaching peak activity after a longer period of asphyxia. There was also a tendency for RSNA to increase higher above baseline levels in near-term compared with preterm fetal sheep. In addition, near-term fetal sheep showed a more rapid rise in BP, and a more rapid fall in RBF and RVC in the first minute of occlusion. Although RSNA increased in all fetal sheep, this increase was delayed until after the initial chemoreflex-mediated fall in HR in the first 30 s of occlusion, and RVC fell markedly well before the large increase in RSNA. These data strongly suggest that RSNA was not a major contributor to the early changes in RBF or RVC. Consistent with this, unilateral denervation in the preterm fetal sheep and chemical sympathectomy in near-term fetal sheep suggest no apparent large differences in RVC compared with controls in the critical first few minutes of occlusion. Thus, despite the clear, strong RSNA response to asphyxia, these data suggest that nerve activity is not the primary determinant of RVC, even at 0.8 gestation. In contrast, the finding in the present study, and previously (41), that chemical sympathectomy in NT fetuses is associated with systemic hypotension in the first minute of occlusion strongly infers that SNA is a critical mediator of initial vasoconstriction in other “peripheral” vascular beds.

Fetal responses to asphyxia can be divided into three main phases. The first phase is marked by a rapid, chemoreflex-mediated fall in HR (4, 29) that decreases workload on the heart (27) and vasoconstriction in the periphery, redirecting blood flow toward the central organs (40). After ~4 min of asphyxia, there is a gradual loss of peripheral vasoconstriction, with a fall in blood pressure that may compromise blood flow to vital organs (40, 71). After 6 to 8 min, overt hypotension develops in a final, decompensation phase that is highly associated with poor fetal recovery (40).

The current study confirms a maturational increase in the speed and magnitude of the initial chemoreflex-mediated vasomotor responses to occlusion, as shown by a more rapid fall in RBF and RVC and increase in BP, consistent with previous studies (23, 71). Our original hypothesis was that this would be

| Table 1. Fetal arterial pH, blood gases, glucose, and lactate values from near-term, preterm, near-term fetal sheep that underwent unilateral renal denervation (PT denervation) 30 min before (baseline), and during asphyxia |
|---------------------------------|-----------------|
|                                | Baseline       | During Asphyxia |
| pH                             |                 |                 |
| NT                             | 7.37 ± 0.01    | 6.94 ± 0.01*    |
| NT-6-OHDA                      | 7.34 ± 0.01    | 6.92 ± 0.04*    |
| PT                             | 7.38 ± 0.01    | 6.87 ± 0.02*    |
| PT-denervation                 | 7.38 ± 0.01    | 6.88 ± 0.02*    |
| Pco2, mmHg                     |                 |                 |
| NT                             | 45.9 ± 1.3     | 113.8 ± 1.2*    |
| NT-6-OHDA                      | 50.9 ± 0.8#    | 116.1 ± 5.7*    |
| PT                             | 45.1 ± 1.2     | 134.0 ± 8.6*    |
| PT-denervation                 | 45.6 ± 1.4     | 132.5 ± 9.1*    |
| Paco2, mmHg                    |                 |                 |
| NT                             | 20.2 ± 1.0     | 5.3 ± 1.2*      |
| NT-6-OHDA                      | 20.3 ± 1.2     | 8.7 ± 0.5*      |
| PT                             | 23.7 ± 1.6     | 11.5 ± 1.6*     |
| PT-denervation                 | 25.4 ± 1.8     | 9.5 ± 0.9*      |
| Lactate, mmol/l                |                 |                 |
| NT                             | 1.1 ± 0.1      | 5.2 ± 0.8*      |
| NT-6-OHDA                      | 1.0 ± 0.0      | 4.9 ± 0.2*      |
| PT                             | 0.8 ± 0.1      | 5.9 ± 0.4*      |
| PT-denervation                 | 0.8 ± 0.1      | 5.5 ± 0.5*      |
| Glucose, mmol/l                |                 |                 |
| NT                             | 0.9 ± 0.05     | 0.9 ± 0.2       |
| NT-6-OHDA                      | 0.8 ± 0.01     | 0.5 ± 0.1*      |
| PT                             | 1.0 ± 0.1      | 0.8 ± 0.1       |
| PT-denervation                 | 0.9 ± 0.1      | 0.7 ± 0.1       |

Values are expressed as means ± SE. Blood samples were taken during asphyxia at 17 min (preterm, PT, and preterm fetal sheep that underwent unilateral renal denervation, PT denervation), 10 min (near-term, NT), and 12 min (near-term fetuses) treated with 6-hydroxydopamine, NT-6-OHDA. Paco2, fetal arterial partial pressure of CO2; Paco2, and fetal arterial partial pressure of CO2; \( P < 0.01 \) vs. baseline; \#P < 0.05 sympathectomy vs. gestation controls.
related to a larger increase in RSNA in near-term fetal sheep. In all fetal sheep, RSNA increased in both burst frequency and amplitude, and although the increase was more sustained in NT than PT fetuses, the magnitude and rate of increase were highly variable and not significantly different between the two age groups. The sheep is relatively precocial, and as in humans, nephrogenesis is complete before birth (48). Thus, while neural development is equivalent to the full-term human at 120 days gestation (52), renal function will be a little less mature. Further, from 120 days onward, there is an increase in fetal cortisol levels that may affect vascular responses (64). Thus, it will be important in future studies to assess whether there is further maturation of the RSNA and the renal response to RSNA during asphyxia by full term.

During the first minute of occlusion, fetal sheep often had intermittent periods of atrioventricular block (Fig. 3). This is postulated to be either a direct effect of hypoxia (63) or “exuberant” vagal stimulation during intense hypoxia (74). Interestingly, during these periods, multiple bursts of RSNA still occurred. This suggests that in the fetus, as suggested in adults (5, 68), bursts of SNA are generated from central circuits independently of baroreceptor input; however, it is the input of
the baroreceptors that entrain the generated bursts of SNA with the cardiac cycle.

Previous studies have shown blunted renal vasoconstriction following denervation in near-term fetal sheep during moderate hypoxia (60). In the present study, it was striking that in both PT and NT fetal sheep, RSNA did not begin to rise until after the rapid initial fall in HR. Further, particularly in NT fetuses, much of the fall in RVC occurred in the first 50 s before the large rise in RSNA. These data suggest that RSNA is not the primary determinant of RVC during asphyxia in either NT or PT fetal sheep. Strongly supporting this, unilateral renal denervation in a PT group and chemical sympathectomy in a NT group were not associated with any significant effect on RVC or RBF during the phase of the large increase in RSNA. Some limitations must be considered. First, for unclear reasons in the PT fetuses, we were unable to achieve the 90% reductions in norepinephrine content of the denervated kidneys reported by others in adults (25, 65). It is still reasonable to note that despite a 56% reduction in norepinephrine content in the present study, there was no apparent effect on RVC. Because of this limitation, we tested the alternative strategy of chemical sympathectomy in NT fetuses. This group was relatively small, and so we cannot exclude the possibility that there may have been some effect of sympathectomy on RVC responses to asphyxia.

Nevertheless, strikingly, chemical sympathectomy in near-term fetal sheep was associated with an initial fall in arterial BP over the first 30 s, when BP was beginning to rise in intact fetuses (41), with no apparent substantial change in RVC.
Given that there was little effect of 6-OHDA on HR and stroke volume is constrained before birth (33), the fall in blood pressure denotes a loss of the initial increase in total peripheral resistance, which must be dependent on vascular beds other than the renal bed. Chemical sympathectomy has been associated with increased sensitivity to norepinephrine (49), which may well contribute to the lack of a fall in HR after sympathectomy compared with acute adrenergic blockade (42) and could potentially attenuate the effects on vascular tone. Nevertheless, unless hypersensitivity was in some way specific to the renal bed, it cannot explain the net loss of total peripheral tone. This is consistent with evidence at 0.9 of gestation, at which point organ blood flow fell more slowly in the kidney after the start of asphyxia and hypoxia than in other peripheral vascular beds (40, 53). Thus, the kidney does not appear to be a “classic” peripheral resistance organ.

Although RSNA does not appear to drive the changes in RVC during asphyxia, the increase in RSNA may play other roles during asphyxia. For example, increased RSNA may promote renin release (62, 66), and, in turn, activate the renin-angiotensin-aldosterone system, which may, in turn, contribute to the reduction in RVC (26, 34). RSNA can also increase activity of the Na⁺-H⁺ exchanger at the proximal tubule, thereby increasing Na reabsorption and helping to sustain BP (51). RSNA may also affect the renal microcirculation, for example, by selective constriction of afferent over efferent arterioles, limiting natriuresis and diuresis during severe asphyxia (65). Finally, there is evidence of functionally specific subgroups of renal nerve fibers that differentially affect renal vasoconstriction and urinary flow rate that may not be evident during monitoring of the whole renal nerve (24).

Given that RVC falls before RSNA increases significantly, it is probable that other mechanisms must have a greater role in mediating the initial fall in RVC in both preterm and near-term fetal sheep. It is most likely related to the independent response of the adrenal glands to hypoxia before birth (1, 16, 18, 47). Indeed, circulating catecholamines peak after just 2 min of asphyxia, corresponding with the timing of the large changes in RSNA and RBF in the current study (17). Further, circulating norepinephrine and epinephrine levels correlated strongly with renal vascular resistance during hypoxia in near-term fetal sheep (72). A high level of redundancy between neural and adrenal catecholamines is consistent with knockout studies that demonstrate that norepinephrine and epinephrine are essential to fetal survival (69). Finally, there could also be an independent effect of asphyxia on the renal vasculature, as both endothelium-dependent vasoconstriction and intramural generation of endothelin-1 contribute to hypoxic vascular tone in fetal pulmonary arteries (19, 70). Further studies are needed to elucidate the role of these factors in the fetal kidney.

Continued asphyxia. The second key finding of this study was that the increase in RSNA was not sustained and, within 5 min of asphyxia, had returned to baseline levels. The mechanisms underlying the loss of RSNA are not known. It occurs at
a time of profound hypoxia, hypercapnia, and metabolic acidosis (see Table 1). Thus, potentially, the profound central neuronal depression and depolarization during occlusion (10) could lead to failure of brain stem generation of RSNA. Regardless of the specific mechanism, the timing is consistent with observations that vagal activity is not required to maintain fetal bradycardia after the first 2 min of severe hypoxia (4, 15).

Alternatively, because umbilical cord occlusion is a complex insult, involving marked hypercapnia and a rapid reduction in umbilical arterial blood flow, as well as hypoxemia, recruitment of additional reflexes may contribute to the fall in RSNA. For example, a similar, albeit less pronounced, biphasic pattern occurs during partial umbilical cord occlusion at term (31), but not during moderate inhalational hypoxia, despite similar partial pressures of oxygen (29). Consistent with the present study, renal chemoreceptor activity became suppressed after 90 s of renal ischemia in adult rats, although RSNA, per se, was not measured (58). Similarly, Fujii et al. (28) found an initial increase in RSNA at the beginning of renal ischemia in adult rats, followed by partial suppression after 5 min, albeit to 50% above baseline, in contrast with the fall in RSNA below baseline levels during prolonged asphyxia in the present study.

**Perspectives and Significance**

The speed, reproducibility, and vascular bed specificity of the initial vasoconstriction during asphyxia and hypoxia in the fetus (3, 22, 41) led to the hypothesis that the primary driver of the vasoconstriction was the sympathetic nerves (30). Although this is highly likely to be correct for other peripheral beds, the present study shows that there is a significant delay after the start of occlusion before RSNA increases in both preterm and near-term fetal sheep, and local or peripheral sympathectomy has no significant effect on renal vascular...
conductance. Thus, these data suggest that RSNAs is not an essential mediator of the renal vascular responses to asphyxia. The most likely candidate-mediator for the initial renal vascular constriction at the onset of asphyxia is a combination of local vascular responses to hypoxia and catecholamine release from the independently oxygen-sensitive fetal renal medulla. Conversely, these data highlight the hypothesis that RSNAs has another major role, perhaps to favor salt reabsorption through differential local effects on the afferent and efferent renal arterioles, increasing Na\(^{+}\)-H\(^{+}\) exchanger activity in the proximal tubule and augmenting the renin-angiotensin-aldosterone responses to asphyxia. These data help to illuminate the highly specific responses of different vascular beds even to a profound, life-threatening challenge.

GRANTS
The present study was supported by grants from the March of Dimes Birth Defects Foundation, the Health Research Council of New Zealand, the Auckland Medical Research Foundation, and the Lottery Grants Board of New Zealand.

DISCLOSURES
Simon Malpas is a director and Sarah-Jane Guild an employee of Telemetry Research, which provided implantable devices for physiological monitoring.

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