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Review

Substance P in cardiovascular diseases – A bioanalytical review

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ABSTRACT

Substance P has recently received much attention as a mediator of adverse heart remodelling and cardiac inflammation by releasing proinflammatory cytokines and matrix metalloproteases from immune and cardiac mast cells. Based on animal models, Substance P is highly associated with the development of cardiomyopathies, subsequently leading to heart failure. After a brief overview of the pathological role of Substance P in cardiac remodelling and cardiac inflammation, this review summarizes the limited, existing data of Substance P blood levels in adults with cardiovascular diseases, demonstrating a high variability of blood concentrations. The investigation of blood levels led to the conclusion that variability is mainly caused by differences in blood sampling and determination. Furthermore, this review illustrates alternate strategies to investigate human Substance P levels as deeper knowledge of them enables further insights into the potential role of Substance P in cardiovascular diseases.

1. Introduction

The classical mammalian tachykinins, namely Substance P (SP), Neurokinin A, and Neurokinin B, are primarily known by their influential role in the central nervous system (CNS) as neurotransmitters. The biological effects of the tachykinins are mediated by three G-coupled receptors, termed Neurokinin (NK) 1–3 receptors [1]. The inhibition of the NK-1-receptor is the pivot of actual clinical investigations because its endogenous agonist activity against SP renders it the most involved tachykinin in various pathological processes [2].

Initially discovered as a mediator in nociception [3] and neurogenic inflammation [4], the role of SP recently expanded to non-neuronal tissues as an important player in cell-cell communication within the innate immune system and peripheral inflammation [5–7]. Consequentially, SP occurs ubiquitously and was found in blood, breast milk, saliva, cerumen, and various peripheral organs, including heart tissue [8–12]. Besides the modulation of emotional behaviour, anxiety, depression, emesis, pain transmission, viral response and neuroinflammation [13–16], the influence of SP in physiological and pathological processes within the cardiovascular system is the foci of current research.

Detailed molecular mechanisms of SP in the cardiovascular system are broadly reviewed [8,17–20] and therefore should not be the principal subject of the present review. However, there exists a crucial lack of information about the determination of SP blood levels in cardiovascular diseases. Therefore, this review will (1) briefly summarize the role of SP in adverse cardiac remodelling and cardiac inflammation; (2) investigate the determination of SP blood levels in cardiovascular diseases in humans focusing on bioanalytical aspects; and (3) discuss impactful factors that may lead to biased SP levels in blood samples.

2. The pathophysiology of Substance P in cardiac remodelling and cardiac inflammation

SP plays both a beneficial and noxious role in heart pathology [21,22]. The release of SP from nerve fibres between cardiomyocytes and coronary artery endothelial cells during cardiac ischemia/reperfusion triggers an acute protective effect by preventing hypoxic cardiac damage and cardiac cell death by activation of NK-1-receptors on cardiomyocytes [23]. Additionally, cardiac hypoxia led to an upregulation of NK-1-receptors on cardiomyocytes, suggesting an acute survival strategy of the heart [24]. Moreover, SP is an indirect potent vasodilator by releasing NO from endothelial cells, resulting in increased myocardial perfusion and restored contractile function of the left ventricle [25].

Over the long-term, the adverse impacts overweigh the acute positive effects of SP on the heart [21], and this is illustrated in Fig. 1. Adverse cardiac remodelling and cardiac inflammation are mainly driven by SP through activation of cardiac mast cells, which are associated with end-stage cardiomyopathy and myocardial infarction [26]. NK-1-receptor activation on cardiac mast cells induces the release of various proinflammatory cytokines, like tumour-necrosis-factor-α

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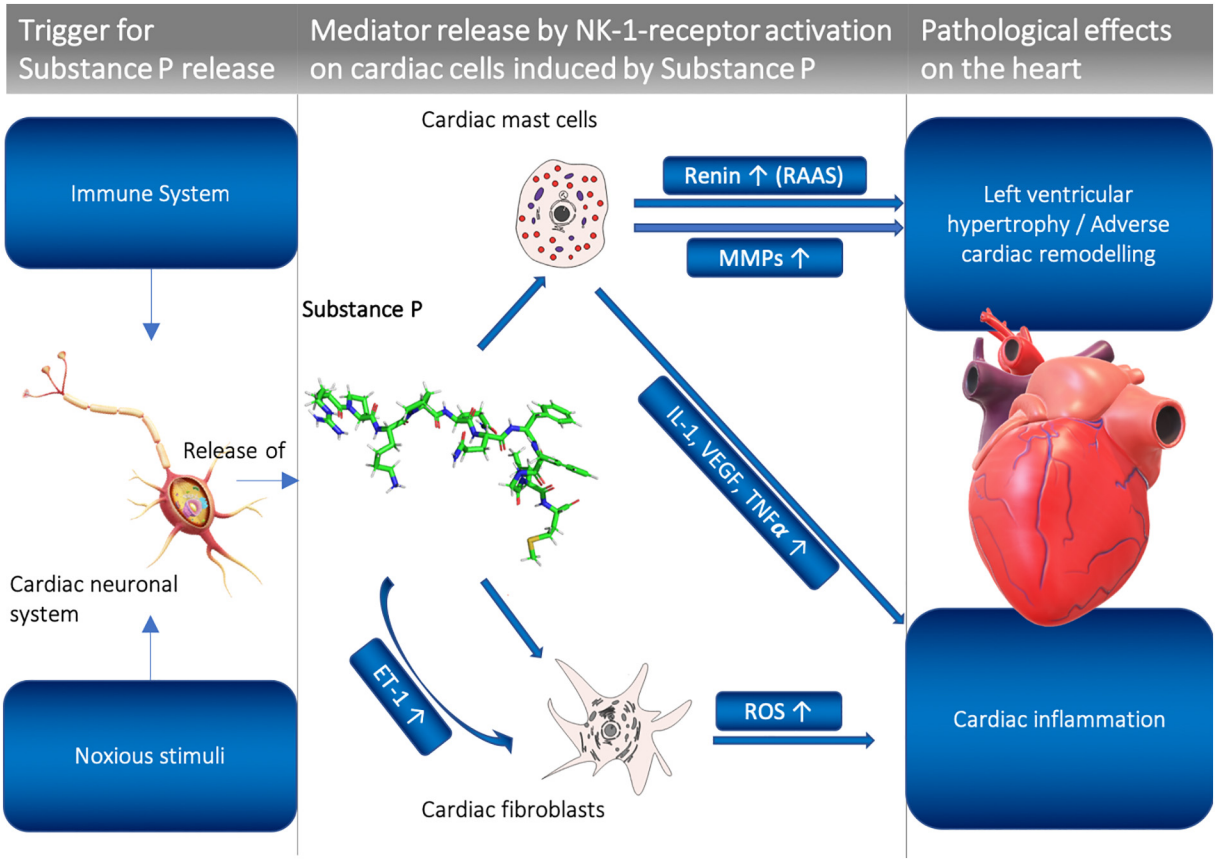

Fig. 1. Molecular scheme of the impact of Substance P in the pathophysiology of cardiovascular diseases (ET-1: endothelin-1, IL-1: interleukin-1, MMP: matrix metalloproteases, NK-1: Neurokinin-1, RAAS: Renin-Angiotensin-Aldosterone-system, ROS: reactive oxygen species, TNF*α*: tumour necrosis factor *α*, VEGF: vascular endothelial growth factor).

(TNFα), interleukins, and matrix metalloproteases (e.g., MMP-2, MMP-9) [27,28]. MMPs are associated with changes in myocardial collagen fibres and fibrillary collagen degradation, leading to adverse cardiac remodelling. So, increased MMP levels were established in dilated ventricles in human heart failure patients and animal myocarditis [29]. In addition, SP induces the release of renin from cardiac mast cells, a protease cleaving Angiotensinogen to Angiotensin-I [30]. Consequently, SP promotes the production of biologically active Angiotensin-II, which is amongst others associated as a mediator of myocardial necrosis [31]. Moreover, SP activates the production of vascular endothelial growth factor in cardiac mast cells, a strong mediator of angiogenesis and releaser of inflammatory cytokines [32]. Coherently, by antagonizing the NK-1-receptor, the degranulation of mast cells and, subsequently, the release of renin, MMPs, and TNFα were inhibited [27]. Furthermore, NK-1-receptor antagonism of cardiac mast cells prevented adverse cardiac remodelling of the left ventricle [28].

Apart from the activation of cardiac mast cells, by upregulating Endothelin-1, SP leads to cardiac fibrosis and activation of foetal genes, which are associated with pathological cardiac hypertrophy [33]. By activation of the NK-1-receptor on cardiac fibroblasts, SP causes cardiac inflammation through the release of reactive oxygen species [34]. In a parasite-infected murine model, SP was moreover associated with compensatory cardiomyocyte hypertrophy, resulting in dilated cardiomyopathy.

In encephalomyocarditis virus (EMCV)-infected mice, > 60-fold higher levels of SP were detected in heart tissue [35]. The virus infection led to high mortality (51%) and significant cardiac hypertrophy in two weeks. On the contrary, SP-precursor knockout mice were completely protected from cardiac inflammation, necrosis, and hypertrophy, all surviving the EMCV-infection. Furthermore, pre-treatment with an NK-1-antagonist significantly reduced mortality and cardiac hypertrophy in EMCV-infected mice [36]. Wang et al. underlined the association of SP with viral myocarditis in EMCV-infected mice [37]. The reasons for the intense SP release in viral myocarditis was not elucidated and invites speculation. Besides an initial immune response owing to viral infection [6] or the aforementioned cardiac survival strategy to prevent myocardial cell damage, there could be also the possibility of molecular mimicry by various viruses [38]. In summary, there is evidence that SP is involved in adverse heart remodelling and the pathogenesis of myocarditis [35], consequentially leading to cardiomyopathy and heart failure.

3. Determination of human Substance P blood levels in cardiovascular diseases

The existing data on SP blood levels in cardiovascular diseases in adults are limited and summarized in Table 1. Data on cardiovascular-diseased children have been lacking until now. First, Valdemarsson et al. (1991) investigated the SP plasma levels in patients with chronic heart failure (CHF), either receiving an Angiotensin-converting enzyme (ACE) inhibitor or not [39]. In their study, mild (NYHA I-II) and severe (NYHA III-IV) CHF were associated with higher SP levels compared to healthy controls (mean ± standard deviation (SD): 4.1 ± 0.4 pg/mL [n = 10; NYHA I-II], 3.1 ± 0.4 pg/mL [n = 17; NYHA III-IV], respectively, vs. 1.7 ± 0.2 pg/mL [n = 31; healthy]), which correlates with the findings of the cell and animal model. As expected, based on the inhibited metabolic degradation of SP, patients undergoing ACE-inhibitor treatment had significantly higher levels of SP (mean ± SD: 5.5 ± 1.1 pg/mL [n = 15]) than untreated diseased subjects (mean ± SD: 3.1 ± 0.4 pg/mL [n = 15]). In 2012, Wang et al. studied

Table 1
Summary of substance P blood levels in cardiovascular-diseased adults

Author (year)	Cardiovascular disease	Subject number	SP mean value* (± SD or IQR) [pg/mL]	Analytical assay (manufacturer)	Matrix	Samplingadditives	LLOQ [pg/mL]	Assay range [pg/mL]	Performance data
Valdemarsson et al. (1991) [39]	Severe CHF under ACEI	15	5.5 (± 1.1)	RIA (n.a.)	Plasma	EDTA (1 mg/mL)	1.3	n.a.	CV (intra-assay precision): < 5%, CV (inter-assay precision): 7.7%
	Severe CHF without ACEI	17	3.1 (± 0.4)						
	Mild CHF without ACEI	10	4.1 (± 0.4)[a]						
	controls	31	1.7 (± 0.2)[a]						
Wang et al. (2012) [40]	Diabetes	46	35 (± 5)[a]	RIA (Huaying Biological engineering / Peninsula Laboratories)	Serum	without additives	1[b]	1 – 640[b]	n.a.
	CAD	44	37.5 (± 6)[a]						
	Controls	44	65 (± 15)[a]						
	Diabetes + CAD	50	15 (± 5)[a]						
Han et al. (2014) [41]	Cardiac infarction	30	191 (± 141)	ELISA (R&D systems)	Serum	Aprotinin (0.6 TIU/mL)	31.5	31.5 – 2500 (R^2: 0.9977)	CV (intra-assay precision): ≤8.4%, CV (inter-assay precision): < 15%, Recovery: 82 – 117%
	Unstable angina	21	103 (± 111)						
	Angina pectoris	30	31 (± 80)						
	Controls	29	70 (± 131)						
Han et al. (2014) [51]	Cardiac infarction	16	215 (median) (100 – 400)[a]	ELISA (R&D systems)	Serum	Aprotinin (0.6 TIU/mL)	31.5	31.5 – 2500 (R^2: 0.9899)	CV (intra-assay precision): ≤8.4%, CV (inter-assay precision): < 15%
	Controls	30	99 (median) (75–125)[a]						
	Cardiac infarction	16	367 (median) (250 – 475)[a]	SQSLISA (in house manufactured)	Serum	Aprotinin (0.6 TIU/mL)	1	1 – 10000 (R^2: 0.9992)	Intra-assay accuracy: 93% [QC: 10 pg/mL], 100% [QC: 100 pg/mL], CV (intra-assay): 7.4% [QC: 10 pg/mL], 4.5% [QC: 100 pg/mL], Inter-assay accuracy: 87% [QC: 10 pg/mL], 122% [QC: 100 pg/mL], CV (inter-assay): 11% [QC: 10 pg/mL], 4.6% [QC: 100 pg/mL]
	Controls	30	95 (median) (50 – 100)[a]						
Vodovar et al. (2015) [42]	ADHF/CHF			RIA (Phoenix Pharmaceuticals)	Plasma	EDTA	10[b]	10 – 1280[b]	n.a.
	With irBNP < 916 pg/mL	440	43 (median) (37 – 49)						
	With irBNP > 916 pg/mL	244	59 (median) (57 – 64)						
Nougué et al. (2018) [43]	CHF			RIA (Phoenix Pharmaceuticals)	Plasma	EDTA	3.8	3.8 – 2000	CV (intra-assay precision): 2.9%, CV (inter-assay precision): 6.3%
	Baseline	73	36 (median) (27 – 44)						
	Under low dose of Sacubitril/Valsartan (49/51 mg)	86	75 (median) (25 – 275)[a]						
	Under high dose of Sacubitril/Valsartan (97/103 mg)	56	100 (median) (50 – 300)[a]						
	After 30 days of treatment	30	75 (median) (50 – 100)[a]						
	After 90 days of treatment	30	175 (median) (75 – 400)[a]						

(n.a.: not available, ACEI: Angiotensin-converting-enzyme-inhibition, ADHF: acute decompensated heart failure, CAD: coronary arterial disease, CHF: chronic heart failure, CV: coefficient of variation, EDTA: ethylenediaminetetraacetic acid, ELISA: enzyme-linked-immunosorbent assay, IQR: interquartile range, irBNP: immunoreactive B-type natriuretic peptide, LLOQ: lower limit of quantification, RIA: radioimmunoassay, SD: standard deviation, SP: Substance P, SQSLISA: silica spheres encapsulating a quantum dot layer-linked immunosorbent assay, TIU: thrombin-inhibition unit; *unless otherwise mentioned; a: estimated values; b: according to the manufacturer)

the SP serum levels in patients with coronary artery disease (CAD) and diabetes [40]. The presence of CAD resulted in decreased SP levels (mean ± SD: 35 ± 5 pg/mL [n = 44]) compared to healthy subjects (mean ± SD: 65 ± 15 pg/mL [n = 44]). In 2014, Han et al. measured SP serum levels in patients with cardiac infarction (mean ± SD: 191 ± 141 pg/mL [n = 30]), unstable angina (mean ± SD: 103 ± 111 pg/mL [n = 21]), stable angina pectoris (mean ± SD: 31 ± 80 pg/mL [n = 30]) and control subjects (mean ± SD: 70 ± 132 pg/mL [n = 29]) [41]. They demonstrated higher SP levels in patients with acute cardiac infarction and suggested the potential role of SP as an early diagnostic biomarker. Based on the metabolic degradation of SP by Neprilysin (NEP), Vodovar et al. (2015) demonstrated the negative correlation of SP and NEP in plasma levels of 684 subjects with acute decompensated heart failure and CHF [42]. Furthermore, SP levels could be significantly stratified regarding immunoreactive B-Type natriuretic peptide (ir-BNP) as a marker for the severity of heart failure (median [interquartile range (IQR)]: 43 [37–49] pg/mL [ir-BNP < 916 pg/mL] vs. 59 [57–64] pg/mL [ir-BNP > 916 pg/mL]). In 2018, Nougué et al. investigated the SP plasma levels of CHF patients under Sacubitril treatment, a selective NEP inhibitor, basically showing higher SP levels under treatment than at baseline (median [IQR]: 75 [25–275] pg/mL vs. 36 [27–44] pg/mL) [43], as expected because of inhibited degradation by NEP.

4. Evaluation of the reliability of Substance P determination in blood samples

As hydrophilic undecapeptide, SP is rapidly metabolized by various degrading peptidases and characterized by a short half-life (approximately 3 min) [44]. Different from the metabolism of SP in the CNS, in blood, SP is dominantly metabolized by three enzymes [45]: the serine protease, Dipeptidyl-peptidase-4 (DPP-IV) [46], and the metalloenzymes, ACE [47] and NEP [42]. These enzymes do not solely inactivate SP but also cleave it in several active metabolites, whereby C-terminal metabolites exhibited a high affinity to the NK-1-receptor [48].

A profound understanding of the metabolism of SP in blood is mandatory to determine accurate SP blood levels (Fig. 2). Investigations of SP metabolites were predominantly executed in CNS tissues, whereby metabolomics play an essential role in the modulation of neuronal functions. In peripheral tissues, SP metabolites seem to play a minor role because of the 100-fold lower SP levels in the blood [49]. Not to be neglected is the metabolic degradation of SP during blood sampling and analysis, potentially resulting in falsified SP levels.

Based on the short metabolic half-life of SP, a mixture of different enzyme inhibitors were introduced to the sampling equipment as required for effective inhibition of SP degradation. In this context, Mosher et al. investigated the degradation of SP with different enzyme inhibitors in terms of sampling equipment and processed the samples at distinct temperature levels [50]. Aprotinin, a serine-protease inhibitor, and ethylenediaminetetraacetic acid (EDTA), a cation chelator inhibiting metalloproteases, are similarly effective in preventing the degradation of SP to commercial broad-spectrum protease inhibitors, which reflect the major degrading enzymes of SP in the blood. Besides, they concluded that not only is the enzyme inhibitor mixture critical, but also the rapid sample procedure after sample collecting. With longer holds of blood samples by ambient temperatures, a substantial loss of SP was observed compared to holding them on ice (approximately 50% in one hour).

Most of the workgroups measuring SP added either Aprotinin or EDTA to the sampling equipment (Table 1). As such, it is remarkable that different workgroups generated distinct SP plasma levels regarding similar collectives. Valdemarsson et al. [39] and Nougué et al. [42] both investigated CHF patients and published significantly different SP plasma levels (mean ± SD: 3.1 ± 0.4 pg/mL vs. median [IR]: 36 [27–44] pg/mL), measured by radioimmunoassay. Besides differences in sample preparation (10 min centrifugation vs. 15 min centrifugation at 4 °C) and sample storage (−20 °C vs. -80 °C), metabolites of SP or structure-related tachykinins may be responsible for imprecise SP levels. Furthermore, the comparison of the same assay technology (immunoassay) using identical samples resulted in distinct SP plasma levels for adults with cardiac infarction (median: 367 pg/mL vs. 215 pg/mL) based on the use of distinct antibodies [51]. The variability of SP blood levels seems to be based on the cross-reactivity and selectivity of the used antibodies, and therefore is examined in greater detail in the following section.

5. Bioanalytical aspects of Substance P determination in blood samples

Determination of SP in blood samples was generally implemented by radioimmunoassays and enzyme-linked-immunoassays. In clinical trials and bioanalytical investigations, the use of immunoassays is common because of advantages in automatization of work procedures and the non-requirement of specialized trained personnel. Nevertheless, immunoassays feature several limitations concerning the determination of SP [52]. The selectivity of immunoassays, especially cross-reactivity to bioactive metabolites of SP, still remains a challenge and depends on the used antibody. Furthermore, with the discovery of HK-1 in 2000 [53], a tachykinin exhibiting > 70% primary amino acid analogy to SP [54] and possessing the same pharmacological active binding site [55], classical immunoassays face the limits of meaningful results. C-terminal targeting antibodies used in immunoassays are not only blind to differentiating between SP and its bioactive C-terminal metabolites, but also between SP and HK-1, biased values being the consequence. As HK-1 is also involved in heart physiology [56,57], a selective determination of SP is mandatory to evaluate the specific role of SP in the cardiovascular system, particularly as reported HK-1 blood levels were substantially higher (> 10-fold) than SP blood levels [58].

An alternative approach for larger diagnostic laboratories could be the use of liquid chromatography coupled to mass spectrometry (LC-MS). The application of LC-MS would enable the determination of peptides, like SP, with sufficient sensitivity according to the advantages of high selectivity and simultaneous measurement of (bioactive) metabolites [59]. So far, several working groups quantified SP concentrations by LC-MS, but they were merely SP levels in various CNS

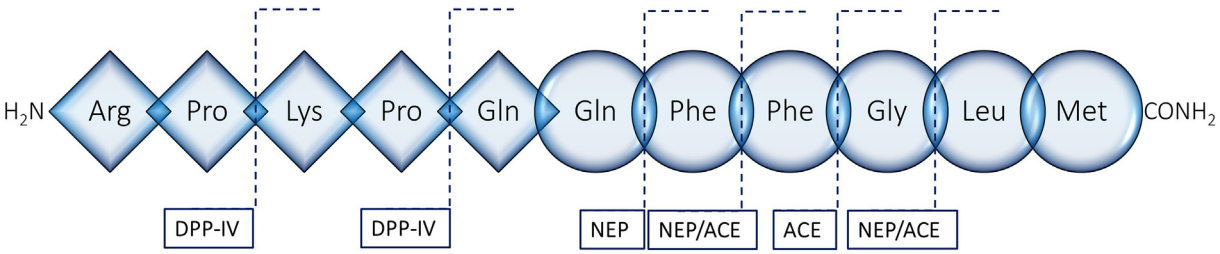

Fig. 2. Primary amino acid structure of Substance P in three-letter-code and its metabolic degradation by Dipeptidyl-peptidase-IV (DPP-IV), Angiotensin-converting enzyme (ACE) and Neprilysin (NEP) in blood samples [active binding site of Substance P to the Neurokinin-1-receptor is illustrated as circles].

tissues in animals [44,49,60,61]. To the authors' knowledge, there exists only a generic approach to quantifying SP in blood samples by LC-MS, but the sensitivity of this approach (limit of detection: 67.4 pg/mL) was not sufficient to measure endogenous SP blood levels [62].

6. Determination of Substance P saliva levels

Focusing on the necessary sensitivity of an analytical assay and limited access to blood, an interesting clinical approach to quantifying SP in a minimally invasive way would be the determination of SP saliva levels. Through the variability of SP levels in saliva, Jang et al. demonstrated the significant correlation of SP in saliva (mean ± SD: 236.1 ± 114.1 pg/mL vs. 177.5 ± 106.2 pg/mL) and plasma (mean ± SD: 168.8 ± 89.5 pg/mL vs. 105.0 ± 67.1 pg/mL) samples of adult chronic migraine patients and control subjects [63]. Besides this, Sato et al. also showed a correlation of SP in human saliva (mean area under the curve (AUC) ± SD: 2394 ± 864.4 ng*min/mL) and plasma (mean AUC ± SD: 5060 ± 1271.3 ng*min/mL) samples [64]. Regarding these promising preliminary results, the correlation between SP saliva and plasma levels needs further investigation to potentially offer a minimally invasive, painless sampling method to evaluate SP levels, especially in severely cardiovascular-diseased patients.

7. Conclusion

Current research into the pathophysiology of cardiomyopathy suggests that SP plays an influential role in adverse cardiac remodelling and cardiac inflammation. The scarcity of effective treatment of cardiomyopathies leads to interest in assessing the role of SP in cardiomyopathy-diseased subjects. Existing data on SP blood levels in cardiovascular-diseased humans features high variability based on differences in sampling and analytical methods. Strict standardization of blood sampling and selective determination of SP levels (by e.g., LC-MS) facilitates the collection of meaningful and reliable data sets.

Declarations of interest

None

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