Association of Plasma Nucleotide levels with severity of Coronary Arterial lesion and left ventricular systolic Function in Acute Myocardial infarction

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ABSTRACT

Background: Purinergic receptors can be divided into adenosine (P1) and adenosine 5'-triphosphate (ATP) (P2) receptors, which play crucial roles in regulating vessel tone, arthrosclerosis, myocardial preconditioning and heart function. However, the relationship of plasma nucleotides with coronary artery disease (CAD) and heart function have not been reported. **Methods and results:** Total 97 patients undergoing coronary angiogram had been tested for the plasma nucleotides, including ATP, uridine 5'-triphosphate (UTP) and adenosine monophosphate (AMP), and their association with CAD and heart function. They were divided into ST-elevation myocardial infarction (STEMI, n=33) group, unstable angina (UA, n=33) group and control (normal angiography, n=31) group. The UTP (1263.23 ± 137.76 vs. 635.61 ± 124.82 nmol/L, P<0.05), ATP (2750.38 ± 215.52 vs. 1903.33 ± 478.80 nmol/L, P<0.05) and AMP levels (1771.06 ± 165.86 vs. 1050.15 ± 124.58 nmol/L, P<0.05) in STEMI group were significantly higher compared to baseline. However, ATP level in UA group was significantly lower than control group (335.33 ± 79.49 vs. 1903.33 ± 478.80 nmol/L, P<0.05). The UTP level had positive correlation with cardiac enzymes and the severity of coronary arterial lesion by Gensini score. Particularly, the plasma UTP level had negative correlation with post-infarction left ventricular ejection fraction (LVEF) within 24 hours (r^2=0.46, P<0.01). **Conclusion:** Plasma nucleotides levels have significant association with the myocardial damage, severity of coronary arterial lesion and left ventricular systolic function in acute myocardial infarction.

Key words: Coronary artery disease, Left ventricular systolic function, Purinergic receptor, ST-elevation myocardial infarction, Unstable angina.

INTRODUCTION

Purinergic receptor was discovered in 1978, which divided into adenosine receptor (P1 receptor) and adenosine 5'-triphosphate (ATP) receptor (P2 receptor). P2 receptor can be sub-divided into P2X (ligand-gated ion channel) and P2Y (G-protein coupled) receptors.[1] Based on pharmacological profile, $P2Y_{1,11,12,13}$ receptors are activated by ATP.[2] It is recognized that uridine 5'-triphosphate (UTP) and uridine 5'-diphosphate (UDP)are equipotent or more potent in $P2Y_{2,4,6,1}4$ receptors.[3] $P2Y_1$, $P2Y_2$, P2Y4, $P2Y_6$ and $P2Y_{11}$ receptors are coupled to G_q, which activate phospholipase C (PLC) and mobilize intracellular calcium signaling. $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ are coupled to G_i, which inhibitsadenylate cyclase,4 hence, decrease intracellular cyclic adenosine monophosphate (cAMP). All P2Y and P2X receptors have been detected at mRNA level in left ventricular myocardium from congestive heart failure (CHF) patients.[5] Among them, the expression and importance of $P2X_7$ receptor have been confirmed in both cardiomyoctes and endothelial cells from mouse heart.[6]

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DOI: 10.5530/jcdr.2015.2.3

Adenosine monophosphate (AMP) has been known to be a potent dilator of coronary artery since 1929,[7] as well as ATP/ADP, which implying the presence of P1 and P2 receptors. $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptors have been identified in cardiac microvascular endothelial cells, as well as $P2X_1$ receptor.[2] For many years, the source of ATP in tissue is considered from damaged cell or exocytotic release from the nerve ending as neurotransmitter.[8] However, it is known presently that many cell types release ATP physiologically in response to hypoxia, stress, exogenous stimulation through channels on the cell membrane, such as connexin or pannexin.[9] ATP can be further breakdown by various ecto-nucleotidase generating ADP or AMP, which formed a sophisticated homeostatic system. Impaired function of endothelial cells was associated with desensitization of P2Y-mediated signaling pathway, which increased nucleotides level in compensatory manner.[10] However, long-term exposure to high level of nucleotides will exacerbate arthrosclerosis.

P2 purinergic receptors are a potential therapeutic target for cardiovascular disease. It was reported that ischemic-reperfusion injury led to release of ATP as a protective response. Either application of exogenous ATP or inhibition of ATP degradation can improve reperfusion injury of coronary endothelial barrier.[11] Erlinge *et al*. reported that UTP level increased early during balloon-induced myocardial ischemia and early after reperfusion. Precondition reduced UTP release, blood flow and ventricular arrhythmias,[12] which may mediated by P2Y2and extracellular regulated protein kinase 1/2 (ERK1/2) pathway.[13,14] In a study enrolled 64 patients, venous plasma levels of UTP were significantly increased in patients with ST-elevation myocardial infarction (STEMI), neither non ST-elevation myocardial infarction (NSTEMI) nor control.[15] Whereas, it is unclear whether the release of nucleotides is a primary or a compensatory process and what is the relationship with cardiovascular disease. Here we demonstrated that plasma nucleotides levels have positive association with the severity of coronary artery disease and left ventricular systolic function.

METHODS

Patient enrollment

This study was approved by Ethics Committee of Peking University Third Hospital and all patients were given written consent to participation in the study. 97 patients undergoing coronary angiogram were recruited in Peking University Third Hospital from Aug 2007 to Oct 2008. It is divided into STEMI group (n=33), unstable angina (UA) group (n=33) and control group (n=31) (atypical chest pain with normal coronary anatomy confirmed by coronary angiography) according to the guideline of hospital and 2004 American College of Cardiology/ American Heart Association guideline.[16] All patients received coronary angiography within 12 hours from symptom **o**nset. 97 patients were enrolled for subgroup studying the relationship of plasma nucleotides and left ventricular systolic function in coronary artery disease (CAD) evaluated by left ventricular ejection function (LVEF). Exclusion criteria were: previous revascularization treatment, transplantation, transfusion in the past 30 days, liver cirrhosis, chronic kidney disease (creatinine>130 μmol/L), anemia (hemoglobin<90 g/L), malignancy, active infection, hyperuricemia, platelet count < 140×109/L. Echocardiogram was performed within 24 hours after coronary angiography by two experienced cardiologists using GE® Vivid 7 echocardiographic machine. LVEF was estimated by Simpson's method with apical two- and four-chamber views. Results of coronary angiogram were interpreted by two experienced interventional cardiologists independently. Gensini score was utilized to evaluate the severity of coronary arterial lesions.[17,18]

Preparation of blood samples and measurement of plasma nucleotides

The concentration of AMP, UTP and ATP in patient plasma was measured as our protocol[19] with modification of protocol.[20,21] Briefly, venous blood was collected at admission (within 2 hours before coronary angiography). All samples were collected in ethylenediaminetetra acetic acid (EDTA)-containing vacutainer tubes and were centrifuged for 10 min (1000×g, 4ºC). Platelet was excluded by Burker chamber examination and the plasma was isolated. One hundred microliters of ice-cold 8% PCA (perchloric acid 70% solution in water) was added into 100 μl plasma. The plasma protein was precipitated after centrifugation (12000×g, 10 min, 4ºC) and the supernatant was collected. 20 μl supernatant was injected into the high performance liquid chromatography (HPLC) system for analysis. The HPLC system was consisted of a quaternary pump (Waters® 2695), a photodiode array detector (Waters® 2996), and a LC-Workstation (Waters® empower). A Hydro-RP C18 analytical column (phenomenex, 250 mm×4.6 mm, 5 μm) was used. The retention time of AMP, UTP and ATP was 11.0 min, 13.9 min and 24.4 min, respectively. The precision of the analytical method, expressed as the intra-day and inter-day relative standard deviation (RSD), was below 4.7% for quality control samples. The accuracy, expressed as the relative error (RE) which contained at least two parallel samples, was within ± 3.9% for all analytes. The

recovery of nucleotide with this analytical method varied from 92% to 109%. The lower limit of quantification (LLOQ) for AMP, UTP and ATP was 0.10, 0.12 and 0.04 μg/ml, respectively.

Statistics

Calculations and statistics were performed using SPSS 15.0. Values were presented as mean ± standard deviation (SD). Student's unpaired *t*-test or one-way analysis of variance (ANOVA) followed by post-hoc test (Tukey's test) was used to determine the significance of differences between comparisons. Pearson or Spearman correlation test and multiple linear regression had been utilized for correlation analysis. Statistical significance was accepted when *P<0.05.

RESULTS

Patients' plasma nucleotides (UTP, ATP and AMP) levels were significantly elevated in STEMI

Total 97 patients were enrolled in this study, including 31 patients in control group, 33 patients in UA group and 33 patients in STEMI group. Baseline characteristics of the patients were comparable (Table 1). In physical exam and laboratory workup, there were significant elevation of cardiac enzymes' peak (creatinine kinase MB, CK-MB and troponin T, TnT), high sensitivity C-reactive protein (hsCRP) and fibrinogen levels in STEMI group. Otherwise other parameters were basically equivalent (Table 2). Patients in STEMI group have significantly elevated plasma concentrations of UTP (STEMI: 1263.23 ± 137.76 vs. Control: 635.61 ± 124.82 nmol/L, P<0.05), ATP (STEMI: 2750.38 ± 215.52 vs. Control: 1903.33 ± 478.80 nmol/L, P<0.05) and AMP (STEMI: 1771.06 ± 165.86 vs. Control: 1050.15 ± 124.58 nmol/L, P<0.05) compared to control group respectively. However, there were no differences between UA and control groups regarding UTP (UA: 822.43 ± 87.18 vs. Control: 635.61 ± 124.82 nmol) and AMP (UA: 896.03 ± 135.06 vs. Control: 1050.15 ± 682.33 nmol/L) levels (P>0.05). ATP concentration was significantly reduced in UA group (UA: 335.33 ± 79.49 vs. Control: 1903.33 ± 478.80 nmol/L, P<0.05) (Figure 1).

Positive correlation between UTP with cardiac enzymes' peak and severity of coronary arterial lesion

Subgroup study in the previous population analyzed plasma

Table 1: Baseline characteristics of patients

Parameters	Control	UA	STEMI	P value
No. of Patients (n)	31	33	33	
Age (years)	63 ±1 2	64 ± 9	65 ± 12	0.07
Sex, Male (%)	16 (51.6)	13 (39.4)	17 (51.5)	0.32
Hypertension (%)	18 (58.06)	26 (78.78)	18 (54.54)	0.07
Diabetes (%)	7 (22.58)	9 (27.27)	8 (24.24)	0.66
Hyperlipidemia (%)	11 (35.48)	12 (36.36)	11 (33.33)	0.79
Smoking history (%)	11 (33.33)	10 (30.30)	17 (51.51)	0.08
Family history (%)	2 (6.45)	1 (3.03)	5 (15.15)	0.09

Table 2: Baseline laboratory and clinical parameters of patients

Parameters	Control (n=31)	UA (n=33)	STEMI (n=33)	P value
Systolic (mmHg)	127.26 ± 21.60	127.06 ± 19.54	126.52 ± 20.16	0.98
Diastolic (mmHg)	79.03 ± 15.29	72.61± 10.34	71.88 ± 11.82	0.05
Gensinis core	0.16 ± 0.52	37.92 ± 28.76	58.18 ± 27.94	0.00*
TC (mmol/L)	4.56 ± 1.01	4.47 ± 0.87	4.29 ± 0.72	0.32
TG (mmol/L)	1.76 ± 0.93	2.10 ± 1.66	1.63 ± 0.85	0.28
HDL-C (mmol/L)	1.07 ± 0.16	1.05 ± 0.22	0.94 ± 0.17	0.05
LDL-C (mmol/L)	2.81 ± 0.89	2.73 ± 0.79	3.19 ± 0.66	0.05
hs-CRP (mmol/L)	0.92 ± 0.76	3.25 ± 8.24	21.32 ± 27.18	0.00*
Fib (g/L)	3.31 ± 0.73	3.58 ± 0.60	3.85 ± 0.87	0.02*
Cr (μmol/L)	97.50 ± 16.00	96.90 ± 17.65	95.57 ± 24.4	0.93
D-Dimer (mg/dl)	0.24 ± 0.20	0.54 ± 1.47	0.36 ± 0.96	0.30
Uric Acid (μmol/L)	334.75 ± 92.13	351.46 ± 78.47	324.27 ± 92.78	0.45
Lp_a (mmol/L)	185.16 ± 237.78	210.09 ± 152.76	196.64 ± 202.13	0.62
CK-MB (U/L) peak	8.32 ± 1.50	11.09 ± 2.66	39.79 ± 6.72	0.00*
TnT (ng/ml) peak	<0.01	<0.01	4.33 ± 0.87	0.00*

*P<0.05 TC, total cholesterol; TG, total triglyceride; HDL-C, high density lipoprotein; LDL-C, low density lipoprotein; hs-CRP, high sensitivity C-reactive protein; Fib, fibrinogen; Cr, creatinine; Lpa, lipoprotein (a); CK-MB, creatine kinase MB; TnT, troponin T

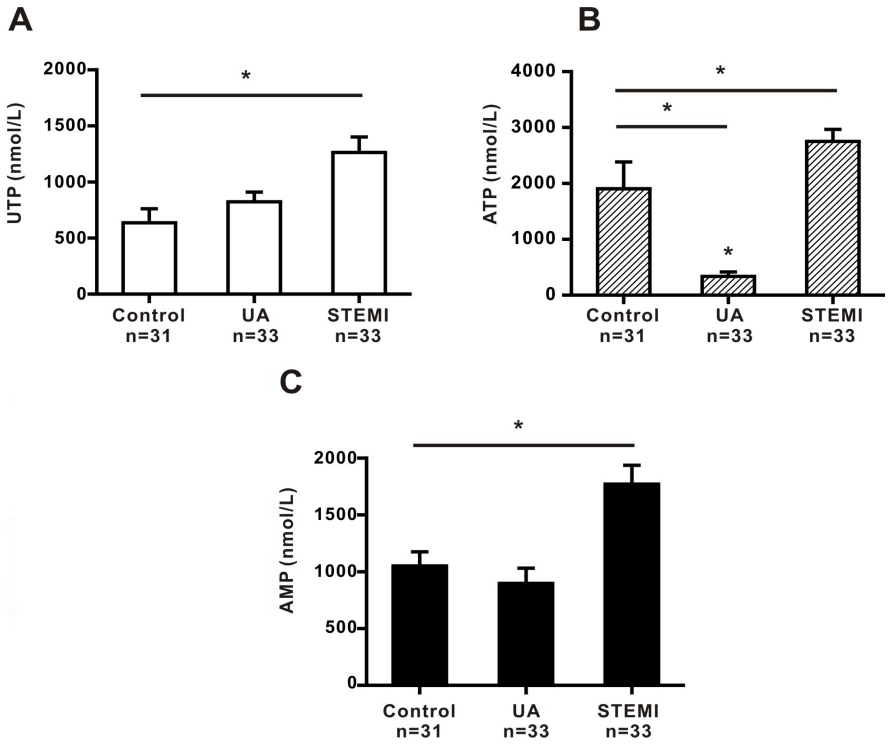

Figure 1: Patients plasma levels of nucleotides, UTP (Figure 1, A), ATP (Figure 1, B) and AMP (Figure 1, C) in control (n=31), unstable angina (UA, n=33) and ST-elevation myocardial infarction (STEMI, n=33) groups expressed as mean value ± SD

*P<0.05 compared with control group

Table 3: Correlation analysis of plasma nucleotides with laboratory parameters

Items	Spearman coefficient (P value)		
	UTP	ATP	AMP
Sex	0.06 (0.59)	-0.11 (0.34)	0.12 (0.31)
Age (years)	0.20 (0.09)	-0.18 (0.10)	0.14 (0.22)
Fib (mg/dL)	-0.10 (0.41)	0.18 (0.12)	0.35 (0.05)
hs-CRP (mg/L)	0.18 (0.14)	0.11 (0.33)	0.31 (0.05)
CK-MB (U/L) peak	0.25* (0.03)	0.20 (0.07)	0.21(0.05)
TnT (ng/mL) peak	0.29* (0.01)	0.24 (0.05)	0.25 (0.05)
Uric Acid (μmol/L)	-0.14 (0.24)	0.01 (0.87)	0.09 (0.41)
D-dimer (μg/mL)	0.01 (0.96)	-0.10 (0.54)	-0.21 (0.21)
Cr (μmol/L)	-0.06 (0.58)	-0.17 (0.13)	-0.03 (0.76)
TC (mmol/L)	0.09 (0.46)	0.21 (0.06)	0.16 (0.16)
TG (mmol/L)	-0.03 (0.81)	0.09 (0.45)	0.06 (0.57)
HDL-C (mmol/L)	0.06 (0.59)	-0.10 (0.35)	-0.14 (0.23)
LDL-C (mmol/L)	0.09 (0.41)	0.07 (0.31)	0.01 (0.18)

*P<0.05. Fib, fibrinogen; hs-CRP, high sensitivity C-reactive protein; CK-MB, creatine kinase MB; TnT, troponin T; Cr, creatinine; TC, total cholesterol; TG, total triglyceride; HDL-C, high density lipoprotein; LDL-C, low density lipoprotein.

Table 4: Plasma nucleotides levels in different coronary artery lesion groups

Parameters	No stenosis Gensini=0 n=29	Moderate stenosis Gensini ≤30 n=22	Severe stenosis Gensini >30 n=46	P value
UTP (nmol/L)	571.25 ± 119.79	916.11 ± 118.61	1389.94±193.4	0.04*
ATP (nmol/L)	1556.99 ± 455.38	1041.51 ± 243.95	1873.21±268.81	0.31
AMP (nmol/L)	1132.84 ± 189.60	1226.02 ± 186.03	1386.40±182.83	0.63

*P<0.05

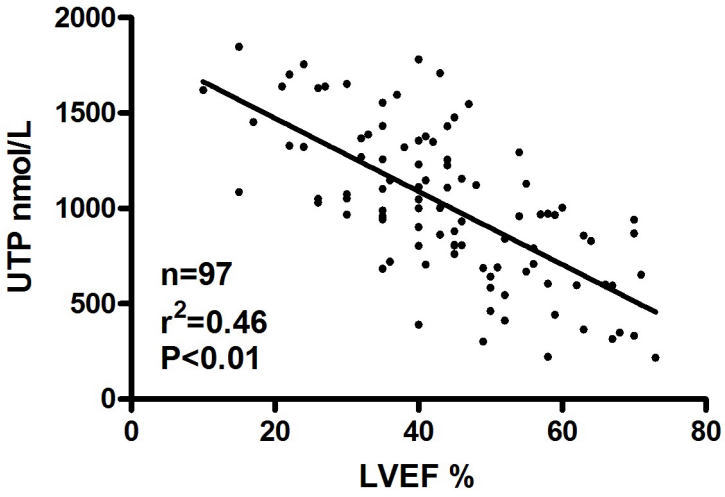

Figure 2: Linear correlation between plasma concentration of UTP and Left Ventricular Ejection Fraction (LVEF) in CAD patients (r^2=0.46, P<0.01, n=97)

nucleotides levels with other laboratory tests showed that UTP had positive correlation with plasma CK-MB and TnT peak levels in STEMI patients (Table 3). Gensini score is a commonly used scoring system to evaluate coronary arterial lesion. Total 97 patients were evaluated, including no stenosis group (Gensini=0, n=29), moderate stenosis group (Gensini≤30, n=22) and severe stenosis group (Gensini>30, n=46). The results demonstrated that UTP also had positive relationship with the severity of coronary arterial lesion (P<0.05). ATP and AMP didn't show significant difference (Table 4).

UTP was associated with 24 hours post infarction LVEF in CAD patients

We further investigated the relation of plasma nucleotides and LVEF in STEMI/UA patients and control subjects. As shown in (Figure 2), linear regression analysis revealed that plasma UTP level had weak linear correlation with LVEF value in 24 hours post infarction (r^2=0.46, P<0.01, n=97). ATP and AMP didn't show significant differences (data not shown).

DISCUSSION

Extracellular nucleotides have been suggested to play important roles in control of cardiovascular pathophysiology, including smooth muscle cells proliferation, regulation of endothelial cells, platelet aggregation, arthrosclerosis and inflammation. Traditionally, plasma nucleotides have five different sources in the body. ATP is released as neurotransmitter from sympathetic nerve ending; ATP and ADP are stored in the dense granules of platelets; adrenal chromaffin cells release nucleotides; vascular smooth muscle cells as well as endothelial cells transport nucleotides from cytosol across the membrane; finally, disruption of cell membrane causes leakage during vascular injury.[22] Previous reports mentioned that the damage of red blood cells during hypoxia can cause ATP increase 2 mmol/L.[23] Hypotonic stress induced 2900 fmol/min ATP release from 1 million bovine aortic endothelial cells.[24] In fact, tissue UTP concentration could be 1000 folds higher than plasma,[12] which may underestimate the real influence of UTP in previous studies.

ATP and other nucleotides can be released by endothelial cell in response to changes in blood flow or vascular tone.[25] In addition, elevation of ATP and UTP were also observed during cardiac ischemia in animal model and served as protective effects.[12,26] Wihlborg *et al.* for the first time reported that UTP level was significantly increased in STEMI patients rather than NSTEMI group.[15] Our study replicates the similar result, indicating extracellular nucleotides could be important factors involving the acute myocardial infarction. We use HPLC to quantify plasma nucleotides in human and the results are equivalent and more accurate compared to previous reports.[12,27,28] In addition, we further demonstrated that plasma nucleotides are not elevated in unstable angina, similarly as the role in NSTEMI.[15] *I*t could be due to non-significant myocardial necrosis in unstable angina/NSTEMI. Therefore, there is no intracellular contents release during this process, which suggesting that plasma nucleotides might be more specific biomarkers for STEMI since it causes severe myocardial death and trans-membranous leakage.

We hypothesize that the significant elevation of ATP level in STEMI might be related to disruption of endothelial and smooth muscle cell membrane, myocardial necrosis or red blood cell damage. Surprisingly, ATP level is significantly decreased in UA patients. The substantial decrement of ATP level might be related to depletion of ATP storage during platelet aggregation in thrombosis without overt myocardial necrosis or activation of NTPase. Platelet contains minor amounts of UTP[29] and therefore UTP is likely from cardiomyocytes.[12,15] Our results further confirm the correlation of UTP with the peak level of cardiac enzymes (CK-MB and TnT), implicating they may be co-released during myocardial necrosis. UTP can be further degraded in to UDP, which is also a potent agonist of $P2Y_6$, $P2Y_2$ and $P2Y_4$ receptors acting as synergistic inotropic effect. However, the product of ATP is adenosine, which counteracts the inotropic effects of ATP. Therefore, UTP could have more potent inotropic cardiovascular effects than ATP.

Our study first demonstrates AMP level in acute coronary syndrome. Although it is the agonist of adenosine A1 receptor, it may indirectly reflect the activity of ecto-nucleoside triphosphate diphosphohydrolase (E-NTPase) and study of the AMP levelcould explore the negative feedback mechanism of purinergic signaling in myocardial infarction. E-NTPase 1 can catalyze ATP and UTP into AMP and UDP respectively. E-NTPase 2 can hydrolyze ATP to ADP, which initiates platelet aggregation and inhibited by E-NTPase 1. The activity and expression of E-NTPase 1 was reported significantly enhanced during acute myocardial infarction,[30] which may illustrate the phenomena of AMP elevation in STEMI group. The competitive inhibition between E-NTPase 1 and E-NTPase 2 would protect over-activation of platelet through depletion storage of ATP.

P2 purinergic receptor knockout mice have been shown resistant to multiple chronic inflammatory disease, such as arthrosclerosis, asthma, or graft-versus-host disease.[31] Stimulation of $P2Y_2$ receptor up-regulated vascular cell adhesion molecule 1 (VCAM1)-mediated macrophage infiltration.[32] Deletion of P2Y1 receptor significantly reduced atherosclerosis in apolipoprotein E (ApoE)-/- mice.[33] Silencing $P2Y_2$ or $P2X_7$ receptor protected cardiomyoctes from hypoxia/ischemia stress.[26] Inhibition or knockout $P2X_7$ receptor showed improvement on long-term allograft survival in cardiac transplant animal model.[34] All of these results indicate nucleotides also involved in the chronic inflammation which facilitates disease progression, including arthrosclerosis and ischemic injury. Our study validated that high UTP level had positive correlation with high Gensini score, which is a commonly used score system evaluating the severity of coronary arterial lesion.[17,18] Furthermore, our study first investigated the relationship between nucleotides levels and left ventricular systolic function in CAD patients. UTP had negative correlation with LVEF in 24 hours (acute) post infarction. This result might indicate that high UTP level is a poor prognostic marker for heart failure which is induced by ischemia. Elevated UTP level could be a compensatory mechanism via activating myocardial $P2Y_6$ receptor and subsequently induces its positive inotropic effect.[15] However, long-term application of UTP induced myocardial hypertrophy and fibrosis through ERK 1/2 signal pathway,[13,32] Similarly, the positive inotropic effect of ATP was also observed in the human cardiac atrium[35] and the effect of pro-apoptosis on murine cardiomyocytes.[36] Chronic exposure and high extracellular nucleotides concentration may lead to desensitization or dysregulation of P2 receptor signaling, therefore, the effect of long term effect of plasma nucleotides need to be further investigated. Our preliminary results show that the plasma UTP level is decreased in end stage heart failure (data not shown).

LIMITATIONS

This study has several limitations. First, its retrospective nature and relative small sample size may have affected the result via selection and other biases. Second, this study cannot illustrate causality, rather than showing associative relationship, therefore the mechanism of nucleotides release is still unclear. Third, the circadian change of plasma nucleotides is not excluded, due to the timing of blood drawing[37] and the effect of pre-medical treatment is another confounding factor which may need further investigation. Fourth, due to the technique difficulties and the inconsistent results, the data of ADP level was not included in this manuscript. Further tests were ongoing and may be published in the near future as well as the effect of purinergic receptor mutation.

CONCLUSION

In conclusion, patients with STEMI have higher plasma levels of nucleotides and UTP level has relationship with severity of coronary lesion, cardiac enzymes' peak and post infarction LVEF values in 24 hours. We provide a novel observation in the relationship of plasma nucleotides and coronary artery disease. To date, three $P2Y_{12}$ receptor inhibitors, clopidogrel, prasugrel and ticagrelor, have been marketed as anti-platelet agents which are all included in current guideline *t*o treat acute myocardial infarction.[38]

Further studies of plasma concentrations of nucleotides and associated purinergic receptor open up new avenues for research and show diagnostic and therapeutic potential for the future treatment of cardiovascular disorders.

ACKNOWLEDGMENT

This project was supported by grant from National Natural Science Foundation of China (81370317).

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