attenuated the progression of GN; the level of GN was reduced from 44.9% to 12.2% and the incidence of GN was reduced from 100% to 36.4%. Furthermore, as indicated, the pre-induction of HIF-1α actually affects the inhibition of GN, because the rate of HIF-1α induction was parallel with that of the attenuation of GN. Therefore, our data suggest that HIF-1α is involved, at least in part, in the defense mechanism against the progression of GN, and hence could be a marker for renal protection.

AII is reported to induce HIF-1α [14,15] and it plays a partial role in the renal protective effect; however, the other effects of AII, such as increasing glomerular pressure and modulating gene expression involving in the renal failure, may overcome any protective effect of AII-induced HIF-1α, and so as a result it may lead to the progression of GN.

In conclusion, we developed a highly reproducible GN model by combining HV and AII. Pre-induction of HIF-1α remarkably attenuated the progression of GN, indicating that HIF-1α was involved in the defense mechanism of the kidney.

Acknowledgments

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References

1 Rifai A, Small PA Jr, Teague PO, Ayoub EM: Experimental IgA nephropathy. J Exp Med 1979; 150: 1161-1173.

2 Ishizaki M, Masuda Y, Fukuda Y, Yamanaka N, Masugi Y, Shichinohe K, Nakama K: Renal lesions in a strain of spontaneously diabetic WBN/Kob rats. Acta Diabetol Lat 1987; 24: 27-35.

3 Banks KL: Glomerulonephritis, autoimmunity, autoantibody. Animal model: anti-glomerular basement membrane antibody in horses. Am J Pathol 1979; 94: 443-446.

4 Elzinga LW, Rosen S, Bennett WM: Dissociation of glomerular filtration rate from tubulointerstitial fibrosis in experimental chronic cyclosporine nephropathy: role of sodium intake. J Am Soc Nephrol 1993; 4: 214-221.

5 Arendshorst WJ, Finn WF, Gottschalk CW: Pathogenesis of acute renal failure following temporary renal ischemia in the rat. Circ Res 1975; 37: 558-568.

6 Wilson CB, Dixon FJ: Immunopathologic mechanisms of renal disease. Ric Clin Lab 1975; 5: 17-38.

7 Masuda Y, Shimizu A, Mori T, Ishiwata T, Kitamura H, Ohashi R, Ishizaki M, Asano G, Sugisaki Y, Yamanaka N: Vascular endothelial growth factor enhances glomerular capillary repair and accelerates resolution of experimentally induced glomerulonephritis. Am J Pathol 2001; 159: 599-608.

8 Kim S, Iwao H: Molecular and cellular mechanisms of angiotensin II-mediated cardiovascular and renal diseases. Pharmacol Rev 2000; 52: 11-34.

9 Lee LK, Meyer TW, Pollock AS, Lovett DH: Endothelial cell injury initiates glomerular sclerosis in the rat remnant kidney. J Clin Invest 1995; 96: 953-964.

10 Huang LE, Arany Z, Livingston DM, Bunn HF: Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its alpha subunit. J Biol Chem 1996; 271: 32253-32259.

11 Wang, GL, Jiang BH, Rue EA, and Semenza GL: Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc Natl Acad Sci USA 1995; 92: 5510-5514,

12 Rosenberger C, Mandriota S, Jurgensen JS, Wiesener MS, Horstrup JH, Frei U, Ratcliffe PJ, Maxwell PH, Bachmann S, Eckardt KU: Expression of hypoxia-inducible factor-1alpha and -2alpha in hypoxic and ischemic rat kidneys. J Am Soc Nephrol 2002; 13: 1721-1732.

13 Wenger RH, Rolfs A, Marti HH, Guenet JL, and Gassmann M: Nucleotide sequence, chromosomal assignment and mRNA expression of mouse hypoxia-inducible factor-1 alpha. Biochem Biophys Res Commun 1996; 223: 54-59,

14 Richard DE, Berra E, Pouyssegur J: Nonhypoxic pathway mediates the induction of hypoxia-inducible factor 1alpha in vascular smooth muscle cells. J Biol Chem 2000; 275: 26765-26771.

15 Page EL, Robitaille GA, Pouyssegur J, Richard DE: Induction of hypoxia-inducible factor-1alpha by transcriptional and translational mechanisms. J Biol Chem 2002; 277: 48403-48409.

16 Raij L, Azar S, Keane W: Mesangial immune injury, hypertension, and progressive glomerular damage in Dahl rats. Kidney Int 1984; 26: 137-143.

17 Linas SL, Shanley PF, Whittenburg D, Berger E, Repine JE: Neutrophils accentuate ischemia-reperfusion injury in isolated perfused rat kidneys. Am J Physiol 1988; 255: F728-F735.

18 Romero F, Rodriguez-Iturbe B, Parra G, Gonzalez L, Herrera-Acosta J, Tapia E: Mycophenolate mofetil prevents the progressive renal failure induced by 5/6 renal ablation in rats. Kidney Int 1999; 55: 945-955.

19 Kaneko Y, Shiozawa S, Hora K, Nakazawa K: Glomerulosclerosis develops in Thy-1 nephritis under persistent accumulation of macrophages. Pathol Int 2003; 53: 507-517.

20 Barnes JL, Lisa MS: Origin of interstitial fibroblasts in an accelerated model of angiotensin II (AII)-induced interstitial fibrosis. J Am Soc Nephrol 2001; 12: 699A3645.

21 Cattell V, Bradfield JW: Focal mesangial proliferative glomerulonephritis in the rat caused by habu snake venom. A morphologic study. Am J Pathol 1977; 87: 511-524.

22 Kitamura H, Sugisaki Y, Yamanaka N: Endothelial regeneration during the repair process following Habu-snake venom induced glomerular injury. Virchows Arch. 1995; 427: 195-204.

23 Ruggenenti P: Angiotensin-converting enzyme inhibition and angiotensin II antagonism in nondiabetic chronic nephropathies. Semin Nephrol 2004; 24: 158-167.

24 Tolins JP, Raij L: Effects of amino acid infusion on renal hemodynamics. Role of endothelium-derived relaxing factor. Hypertension 1991; 17: 1045-1051.

25 Nakamura T, Obata J, Kimura H, Ohno S, Yoshida Y, Kawachi H, Shimizu F: Blocking angiotensin II ameliorates proteinuria and glomerular lesions in progressive mesangioproliferative glomerulonephritis. Kidney Int 1999; 55: 877-889.

26 Makino Y, Cao R, Svensson K, Bertilsson G, Asman M, Tanaka H, Cao Y, Berkenstam A, Poellinger L: Inhibitory PAS domain protein is a negative regulator of hypoxia-inducible gene expression. Nature 2001; 414: 550-554.

27 Neckers LM: aHIF: the missing link between HIF-1 and VHL? J Natl Cancer Inst 1999; 91: 106-107.

28 Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER, Ratcliffe PJ: The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature 1999; 399: 271-275.

29 Zhou J, Fandrey J, Schumann J, Tiegs G, Brune B: NO and TNF-alpha released from activated macrophages stabilize HIF-1alpha in resting tubular LLC-PK1 cells. Am J Physiol Cell Physiol 2003; 284: C439-C446.

30 Sandau KB, Zhou J, Kietzmann T, Brune B: Regulation of the hypoxia-inducible factor 1alpha by the inflammatory mediators nitric oxide and tumor necrosis factor-alpha in contrast to desferroxamine and phenylarsine oxide. J Biol Chem 2001; 276: 39805-39811.

31 Prass K, Ruscher K, Karsch M, Isaev N, Megow D, Priller J, Scharff A, Dirnagl U, Meisel A: Desferrioxamine induces delayed tolerance against cerebral ischemia in vivo and in vitro. J Cereb Blood Flow Metab 2002; 22: 520-525.

32 Furuta GT, Turner JR, Taylor CT, Hershberg RM, Comerford K, Narravula S, Podolsky DK, Colgan SP: Hypoxia-inducible factor 1-dependent induction of intestinal trefoil factor protects barrier function during hypoxia. J Exp Med 2001; 193: 1027-1034.

33 Matsumoto M, Makino Y, Tanaka T, Tanaka H, Ishizaka N, Noiri E, Fujita T, Nangaku M: Induction of renoprotective gene expression by cobalt ameliorates ischemic injury of the kidney in rats. J Am Soc Nephrol 2003; 14: 1825-1832.

Figure Legends

Fig. 1. Study Protocol. All rats are unilaterally nephrectomized on day -1 and divided into 4 groups on day 0.
N group; no injection of reagents. HV group; injection of 3.5mg/kg of Habu snake venom (HV). A group; continuous administration of 100ng/min of Angiotensin II (AII). H+A group; administration of HV and AII.

Fig. 2. Glomerulonephritis is developed with the combination of HV and AII, and HIF-1α is induced in the intact glomeruli.
There are no glomerular or tubular injuries in N group (A), HV group (B), A group (C) and H+A group on day 1 (D). Damaged glomeruli, characterized by extensive mesangiolysis, are observed in H+A group on day 2. PAS staining. Magnification, *100 (E). Focal and segmental mesangiolysis with large capillary aneurysmal ballooning are observed in the H+A group on day 2. PAM staining. Magnification, *400 (F). The number of GN was significantly less in pre-treatment with $CoCl_2$ than without. PAS staining. Magnification, *100 (G). Immunoreactive HIF-1α positive signals are not detected in the N group (H). Nuclear HIF-1α signals are observed in a glomerulus and tubules in the A group. Magnification, *200 (I). A glomerulus in the H+A group on day 2 possesses intact cells with HIF-1α positive signals, in contrast, other parts have few HIF-1α signals due to mesangiolysis. Magnification, *200 (J).

Fig. 3. Semiquantitative analysis of morphologic changes in our glomerulonephritis model. The main lesion in the H+A group is initially detected on day 2 as mesangiolysis in glomeruli; however, there are no tubular lesions of necrosis except for tubular casts; in contrast, there are no morphological changes in the N and A groups. MES, Mesangiolysis score.

Fig. 4. Serum UN, Cr and SBP are increased with the combination of HV and AII. The serum UN (A) and Cr (B) levels in the H+A group on day 2 are significantly higher than other groups. SBP increases significantly with administration of AII (A and H+A groups) (C).

Fig. 5. The protein level of HIF-1α is increased by administration of HV and AII, and pretreatment of $CoCl_2$ increases HIF-1α expression before development of GN. HIF-1α is not detected in the N and HV groups (Day2). However, HIF-1α is detected in A (Day2) and H+A (Days 1 and 2) groups (A). The $CoCl_2$ group, in accord with the level of HIF-1α induction, was divided into two groups. HIF-1α is greatly induced before the development of GN ($CoCl_2$ group Pre-1), and is followed by a high level ($CoCl_2$ group Day2-1); in contrast, it is not efficiently induced ($CoCl_2$ group Pre-2), and also is scarcely detected on day 2 ($CoCl_2$ group Day2-2) (B). The rate of pre-induction of HIF-1α by $CoCl_2$ is comparable with that of the inhibition of GN by $CoCl_2$ (C).

Fig. 6. Pretreatment with $CoCl_2$ attenuates GN. Serum UN (A) and Cr (B) levels in the $CoCl_2$ group on day 2 are significantly decreased compared to those in the non-$CoCl_2$ group. There is no significant difference in SBP between the $CoCl_2$ and non-$CoCl_2$ groups (C).

Fig. 1

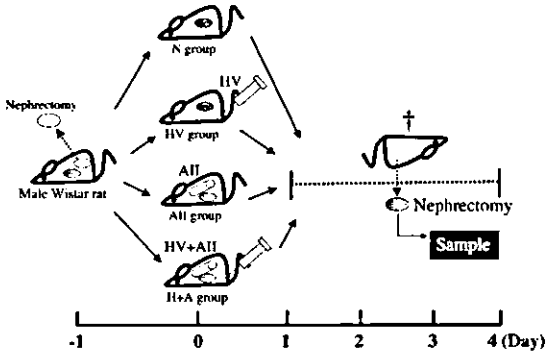


Fig. 2

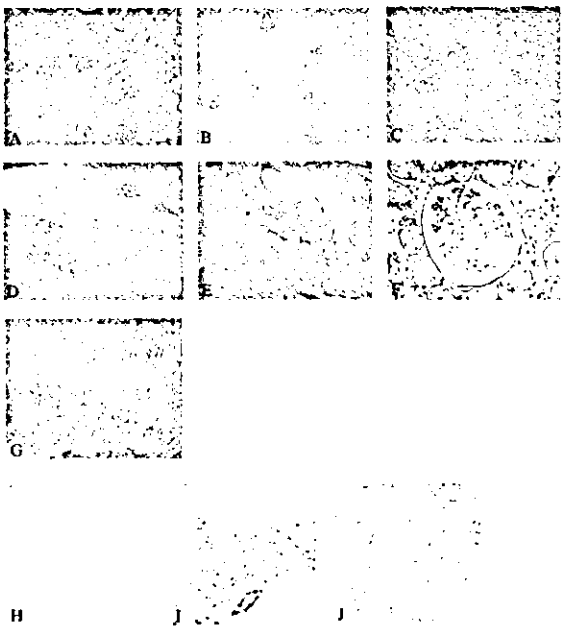
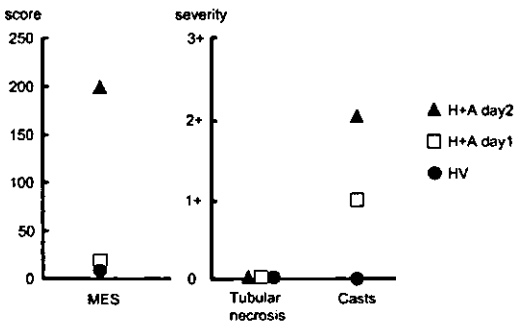


Fig. 3



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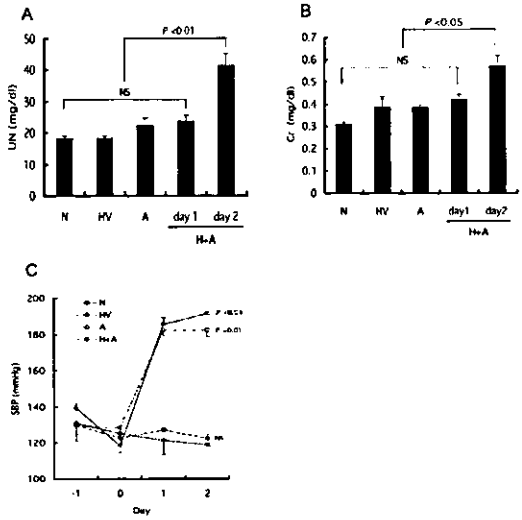


Fig. 4

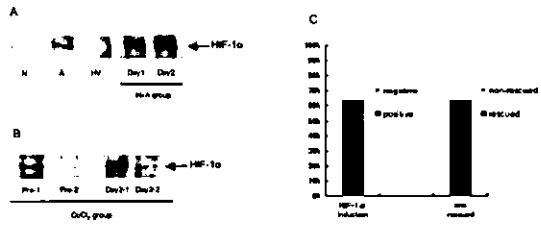
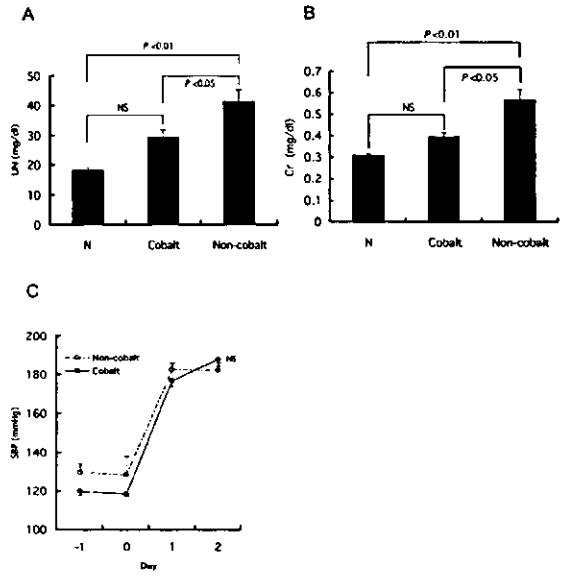


Fig. 5



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ORIGINAL ARTICLE

Association between arterial stiffness and platelet activation

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Increased arterial stiffness is strongly associated with atherosclerosis, while platelet activation is an important trigger of thrombotic events in patients with atherosclerosis. However, little is known about the effect of arterial stiffness on platelet activation. We therefore investigated the association between arterial stiffness and platelet activation in 38 normal volunteers (20 men and 18 women) aged 23–77 years (mean = 49 ± 15 years). Arterial stiffness was assessed by measuring brachial–ankle pulse wave velocity (ba-PWV) and heart–brachial PWV (hb-PWV). Flow cytometric analyses were performed to evaluate platelet activation by measuring surface expression of P-selectin and platelet–neutrophil complexes (PNC) before and after activation by ADP. We also calculated the difference between basal and stimulated states of P-selectin and PNC to assess platelet activation reserve. PWVs were significantly correlated with age and BP ($r = 0.60–0.81$). For platelet activation and activation reserve, correlations with age were less strong but remained significant ($r = 0.36–0.61$), with the exception of P-selectin (not significant, NS), and correlations with SBP were similar ($r = 0.35–0.53$). A significant correlation was found between PWVs and platelet activation ($r = 0.43–0.74$). Multiple regression analysis demonstrated significant correlations between platelet activation and reserve and PWVs (coefficient = 2.17–6.59), when both age and BP were adjusted for simultaneously. In conclusion, platelet activation was associated with arterial stiffness, suggesting that arterial stiffness may play an important role in thrombotic events.

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Keywords: arterial stiffness; pulse wave velocity; P-selectin; platelet–neutrophil complexes

Introduction

Platelet activation and aggregation are important triggers of thrombotic events in patients with atherosclerosis. In such patients, platelets are activated at the site of atheroma due to increased shear stress in the narrowed vessels.[2,3] Increased platelet activation is observed in patients with coronary risk factors and cardiovascular events.[4–12]

Increased arterial stiffness, measured with pulse wave velocity (PWV), has been shown to be associated with atherosclerosis and risk factors of atherosclerotic cardiovascular disease,[13–21] and is an independent predictor of cardiovascular events.[22,23] Therefore, although platelets are likely to be activated in patients with atherosclerotic disease who exhibit increased arterial stiffness, little is known about the relation of arterial stiffness itself to platelet activation.

Recently, platelet activation has been widely evaluated by measuring soluble P-selectin; a platelet surface molecule also termed CD62P.[4,6–8,11] Although the measurement of soluble P-selectin is simple and useful, it is an indirect method of evaluating platelet activation. On the other hand, platelet activation can be detected directly by measuring surface antigen CD62P using flow cytometry.[2,3,5,9,10,12] Furthermore, detection of platelet–neutrophil complexes (PNC), which are formed as a result of interaction with CD62P provides an additional means to detect platelet activation.[24]

The purpose of this study was to investigate the association between arterial stiffness and platelet activation by measuring PWV, P-selectin, and PNC in subjects without atherosclerotic disease.

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Materials and methods

Subjects

We studied 38 healthy nonsmoking volunteers (20 men and 18 women), aged 23–77 years (mean = 49 ± 15 years) with no evidence of heart disease on physical examination, standard 12-lead electrocardiography, chest radiography, echocardiography, or blood chemistry analysis. Subjects had no self-reported past history or current evidence of cardiovascular disease, hypertension, hypercholesterolaemia, diabetes mellitus or renal disease. Basic characteristics of subjects are shown in Table 1. None of the subjects had frequent ectopic beats or atrial fibrillation and none had taken any medication for at least 10 days. Informed consent was obtained before performing the study and the study protocol was approved by the Local Ethics Committee of Kochi Medical School.

Evaluation of arterial stiffness

Arterial stiffness was evaluated by PWV, measured using volume-plethysmographic apparatus (Colin, Komaki, Japan).[18–21] Data were acquired with subjects lying supine in a quiet and temperature-controlled room at 11 AM, at least 3 h after breakfast. Surface electrodes were attached to both wrists for ECG measurement, a microphone was positioned at the left sternal edge to detect heart sounds, and cuffs incorporating plethysmographic and oscillometric sensors were fastened around both the brachial regions and ankles to measure pulse wave forms and blood pressure. Brachial–ankle PWV (ba-PWV) and heart–brachial PWV (hb-PWV) were measured as follows. The time interval between the wave foot of the brachial waveform and that of the ankle waveform was defined as the time interval between the brachial region and ankle, while the time interval between the heart and the right brachial artery was defined as the time interval between the second heart sound and the right brachial waveform. The distance between these sampling points was calculated automatically according to the height of the subject. PWVs were calculated by dividing each distance by the respective time interval. Right brachial blood pressure (systolic and diastolic) and pulse rate were concurrently measured.

Table 1 Clinical characteristics of subjects

Parameters	*All subjects (n = 38)*
Age (years)	49 ± 15
Gender, male/female	20/18
Systolic blood pressure (mmHg)	125 ± 16
Diastolic blood pressure (mmHg)	77 ± 10
Pulse rate (bpm)	66 ± 10
Blood sugar (mg/dl)	98.5 ± 18.5
Total cholesterol (mg/dl)	192.6 ± 20.7
Blood urea nitrogen (mg/dl)	14.0 ± 18.5
Creatinine (mg/dl)	0.69 ± 0.15
PNC (%)	9.5 ± 4.9
PNC(ADP) (%)	20.2 ± 9.9
Δ-PNC	10.7 ± 6.9
P-selectin (%)	13.1 ± 1.7
P-selectin(ADP) (%)	36.6 ± 9.2
Δ-P-selectin	23.6 ± 9.1
hb-PWV (m/s)	5.3 ± 0.9
ba-PWV (m/s)	13.8 ± 3.0

Values are expressed as mean ± s.d.
PNC = platelet neutrophil complexes; ADP = adenosine diphosphate; Δ-PNC = PNC (ADP) − PNC; Δ-P-selectin = P-selectin (ADP) − P-selectin; hb-PWV = heart–brachial pulse wave velocity; ba-PWV = brachial–ankle pulse wave velocity.

Measurement of platelet activation

Sample preparation and measurement of platelet P-selectin (CD62P) and PNC levels were performed according to the method described by Peters *et al.*[24] To minimize platelet activation during blood collection, blood was drawn via a 21 G butterfly needle without the use of a tourniquet. After discarding the first 2 ml of blood, a further 2 ml was collected and immediately added to 200 μl of sodium citrate (3.1%). All antibodies were sourced as follows: Fluorescein isothiocyanate (FITC) labelled IgG1 anti-CD62P from Dainippon Pharmaceutical, Osaka, Japan, phycoerythin (PE) labelled IgG2a anti-CD42b and FITC labelled IgG1 anti-CD11b from Beckman Coulter, Fullerton, CA, USA. As negative controls, FITC-labelled IgG1 (Beckman Coulter, Fullerton, CA, USA) and double-stained (FITC/PE) IgG1 and IgG2a (Dako, High Wycombe, Bucks, UK) irrelevant antibodies were included.

Sample preparation for the measurement of platelet CD62P level: In all, 5 μl of blood was added to a round-bottomed polystyrene tube containing 50 μl of platelet buffer (10 mmol/l HEPES, 145 mmol/l NaCl, 5 mmol/l KCl, 1 mmol/l $MgSO_4$; pH 7.4), and 5 μl of anti-CD62P or control IgG1 antibody. Following gentle suspension, samples were incubated in the dark at room temperature for 20 min without stirring. Then 250 μl of fixative was added and the tubes were incubated for an additional 10 min. The samples were then diluted with 500 μl of buffer and analysed. Flow cytometric analysis was performed within 1 h of fixation.

Sample preparation for the measurement of PNC level: In all, 50 μl of blood was added to a round-bottomed polystyrene tube containing 5 μl of anti-CD42b, and 5 μl of anti-CD11b or isotype control antibodies. Following gentle mixing, samples were incubated in the dark at room temperature for 10 min without stirring. Then 500 μl of fixative was added and the tubes were incubated for additional 10 min. Flow cytometric analysis was performed within 1 h of preparation.

Flow cytometric analysis

Blood samples were analysed in a COULTER EPICS XL Profile Flow Cytometer, Miami, FL, USA, using either single or double fluorochromes. The peak emission intensity of FITC fluorescence was detected at 515 nm and that of phycoerythin fluorescence at 580 nm.

Measurement of platelet CD62P level: After forward and side scatter measurements were made with gain setting in logarithmic mode, platelet-sized events were counted. CD62P-positive platelets were defined as those with a fluorescence intensity exceeding that of 98% of the platelets staining with control antibody.

Measurement of PNC level: After forward and side scatter measurements were made with gain setting in linear mode, neutrophil-sized events were selected. Results were defined as positive when the fluorescence intensity exceeded that of 98% of the isotype-matched (IgG1 and IgG2a) control antibodies staining. Events positive for both CD11b and CD42b were considered to represent PNCs and were expressed as percentages of events with positive CD11b staining.

Evaluation of platelet activation reserve: We evaluated platelet activation reserve, that is, the ability of the platelets to be activated, in a separate experiment. Platelets were activated with 5 μl of adenosine diphosphate (ADP). We also calculated the difference between basal and stimulated states of P-selectin expression (Δ-P-selectin) and PNC level (Δ-PNC) to determine activation reserve.

Statistical analysis

Data are presented as mean±s.d. Univariate linear correlation analysis and multiple regression analysis were used for statistical evaluation. The variables significantly associated with platelet activation on univariate analysis were included in a multiple regression analysis in order to adjust PWV for each variable. Gender differences were evaluated with ANOVA. *P*-values <0.05 were considered to represent statistical significance.

Results

Both ba-PWV and hb-PWV exhibited significant positive correlations with age, systolic, and diastolic blood pressure ($r=0.60$–0.81, $P<0.05$ or <0.01), and pulse rate ($r=0.44$, $P<0.05$, $r=0.65$, <0.01, respectively) (Table 2). For platelet activation and activation reserve, correlations with age were less strong but remained significant ($r=0.36$–0.61, $P<0.05$ or <0.01) with the exception of Δ-P-selectin (not significant, NS), and correlations with systolic and diastolic blood pressure were similar ($r=0.35$–0.53, $P<0.05$ or <0.01) with the exception of P-selectin (NS) (Table 3). However, platelet activation and activation reserve exhibited no significant correlation with pulse rate, blood glucose, total cholesterol, blood urea nitrogen or creatinine. No significant gender-related differences were observed in any of these correlations (Tables 2 and 3).

Table 2 Correlation between PWV and clinical indices

	hb-PWV	*ba-PWV*
Age	0.74**	0.80**
Systolic blood pressure	0.61**	0.81**
Diastolic blood pressure	0.60**	0.74**
Pulse rate	0.44*	0.65**
Blood sugar	−0.05	−0.17
Total cholesterol	−0.03	−0.30
Blood urea nitrogen	−0.32	0.32
Creatinine	0.04	−0.14
Gender		
Male	5.5±1.0	14.1±3.0
Female	5.2±0.8	13.6±3.1

PNC = platelet neutrophil complexes; ADP = adenosine diphosphate; Δ-PNC = PNC (ADP) − PNC; Δ-P-selectin = P-selectin (ADP) − P-selectin; hb-PWV = heart–brachial pulse wave velocity; ba-PWV = brachial–ankle pulse wave velocity.
For parameters from age to creatinine, values are correlation coefficients.
*$P<0.05$.
**$P<0.01$.
For gender, values are mean±s.d., with differences evaluated with ANOVA.

PWVs exhibited significant positive correlations ($r=0.43$–0.74, $P<0.05$ or <0.01) to all indices of platelet activation and reserve (Table 4, Figure 1). When age or blood pressures were adjusted for on multivariate analysis, some indices of platelet activation and reserve were significantly related to PWVs ($r=0.34$–7.67, $P<0.05$ or <0.01). When both age and blood pressures were simultaneously adjusted for, significant correlations remained between platelet activation and reserve and PWVs ($r=2.17$–6.59, $P<0.05$ or <0.01) (Table 4). In other words, although the relationship between PWVs and the indices of platelet activation was strongly affected by age and blood pressure, a significant association remained when these factors were adjusted for.

Discussion

The main finding of this study was that platelet activation and activation reserve were associated with arterial stiffness when analyses were adjusted for age and blood pressure. This suggests that increased arterial stiffness might play an important role in thrombotic events.

Patients with hypertension, cerebrovascular disease, coronary heart disease, diabetes mellitus, and renal failure are recognized to have less arterial compliance than normal subjects.[13–15,17–19] Increased PWV has also been reported to be an independent predictor of cardiovascular events in patients with

Table 3 Correlation between platelet activation and clinical indices

	PNC	*PNC (ADP)*	*Δ-PNC*	*P-selectin*	*P-selectin (ADP)*	*Δ-P-selectin*
Age	0.51**	0.61**	0.52**	0.36*	0.38*	0.32
Systolic blood pressure	0.41*	0.53**	0.48**	0.41*	0.43*	0.35*
Diastolic blood pressure	0.43*	0.49**	0.40*	0.25	0.40*	0.36*
Pulse rate	0.28	0.25	0.16	0.04	0.15	0.15
Blood sugar	0.09	−0.18	−0.31	−0.17	0.13	0.16
Total cholesterol	−0.14	−0.07	0.001	−0.10	−0.13	−0.11
Blood urea nitrogen	−0.01	0.12	0.18	−0.05	0.05	0.06
Creatinine	0.05	−0.13	−0.22	0.04	−0.17	−0.18
Gender:						
male	10.3±5.9	19.7±8.7	9.4±6.9	13.1±1.8	35.5±9.3	22.4±9.0
female	8.8±3.8	20.7±11.4	11.9±6.8	13.0±1.7	37.7±9.2	24.7±9.3

PNC = platelet neutrophil complexes; ADP = adenosine diphosphate; Δ-PNC = PNC (ADP)−PNC; Δ-P-selectin = P-selectin (ADP)−P-selectin; hb-PWV = heart–brachial pulse wave velocity; ba-PWV = brachial–ankle pulse wave velocity.
For parameters from age to creatinine, values are correlation coefficients.
*$P<0.05$.
**$P<0.01$.
For gender, values are mean±s.d., with differences evaluated with ANOVA.

Table 4 Relation between platelet activations and PWV

	PNC	*PNC (ADP)*	*Δ-PNC*	*P-selectin*	*P-selectin (ADP)*	*Δ-P-selectin*
Not adjusted						
hb-PWV	0.62**	0.74**	0.63**	0.45**	0.57**	0.50**
ba-PWV	0.59**	0.71**	0.61**	0.47**	0.51**	0.43*
Adjusted for age						
hb-PWV	2.86**	6.95**	4.09*	0.75	6.55**	5.80*
ba-PWV	0.79	2.01**	1.22*	0.28	1.75*	1.47
Adjusted for systolic blood pressure						
hb-PWV	3.20**	7.23**	4.04**	0.59	5.09*	4.50*
ba-PWV	1.21**	2.64**	1.44*	0.23	1.48	1.25
Adjusted for diastolic blood pressure						
hb-PWV	3.08**	7.67**	4.59**	0.87*	5.32**	4.46*
ba-PWV	0.97**	2.50**	1.54**	0.34*	1.45*	1.10
Adjusted for age and systolic blood pressure						
hb-PWV	2.80*	6.43**	3.63*	0.58	5.93*	5.35*
ba-PWV	1.08	2.32*	1.24	0.24	1.72	1.48
Adjusted for age and diastolic blood pressure						
hb-PWV	2.63*	6.59**	3.97**	0.78	6.06*	5.28*
ba-PWV	0.76	2.17	1.40	0.40	1.66	1.26

PNC = platelet neutrophil complexes; ADP = adenosine diphosphate; Δ-PNC = PNC (ADP)−PNC; Δ-P-selectin = P-selectin (ADP)−P-selectin; hb-PWV = heart–brachial pulse wave velocity; ba-PWV = brachial–ankle pulse wave velocity.
'Not adjusted' — values are correlation coefficients between PWVs and indices of platelet activation before adjustment.
*$P<0.05$.
**$P<0.01$.
Other values are regression coefficients between PWVs and indices of platelet activation adjusted for age and/or blood pressures as indicated.
*$P<0.05$.
**$P<0.01$.

hypertension or renal failure, and in elderly subjects.[22,23] The association between increased arterial stiffness and high incidence of cardiovascular events may be explained by the existence of atherosclerosis. Hirai *et al*[25] have demonstrated strong associations between abdominal aortic and carotid arterial stiffness and the degree of coronary artery disease. Popele *et al*[26] recently reported that aortic stiffness as measured by PWV is strongly associated with common carotid intima-media thickness, carotid arterial plaques, and the presence of peripheral arterial disease. Moreover, some population-based studies have demonstrated higher blood pressure, increased age, and male gender to be associated with increased PWV.[16,20,21] Pulse pressure may also relate to arterial stiffness and cardiovas-

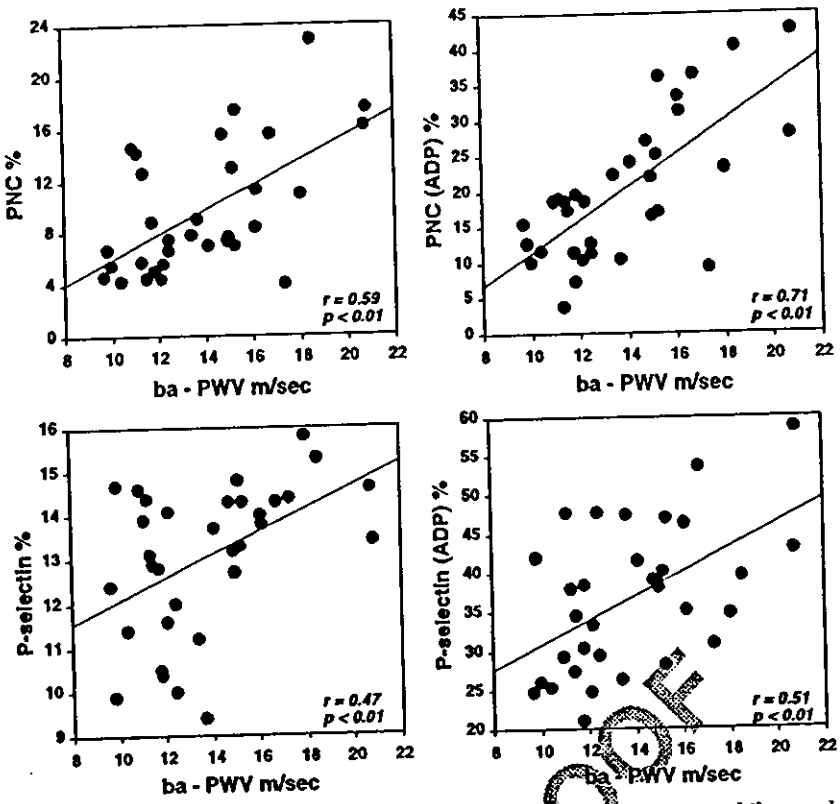

Figure 1 Correlation between ba-PWV and PNC (upper two panels). PNC = platelet neutrophil complexes; ADP = adenosine diphosphate; Δ-PNC = PNC (ADP)–PNC; Δ-P-selectin = P-selectin (ADP)–P-selectin; hb-PWV = heart–brachial pulse wave velocity; ba-PWV = brachial–ankle pulse wave velocity.

cular events, with higher pulse pressure reflecting elevated systolic pressure and reduced diastolic pressure due to increased arterial stiffness. In the present study, significant relationships were observed between PWVs and age, blood pressure, and pulse rate, in accordance with previous studies.

P-selectin is a component of α-granules that is expressed on the platelet surface membrane and released into the plasma upon platelet activation. Although the bulk of circulating soluble P-selectin appears to be platelet derived,[27] the substance is also found in the Weibel–Palade bodies of endothelial cells.[28] Direct measurement of platelet membrane P-selectin is therefore a more sensitive method of assessing platelet activation. In the present study, we evaluated platelet activation by measuring membrane activation markers using flow cytometry with activation-dependent monoclonal antibodies. PNC levels were also measured using the same method.[9] P-selectin levels in our normal subjects aged 49±15 years were 13.1±1.7%; this was higher than that in quoted by other studies, possibly due to the differences in monoclonal antibodies or in sample manipulation.

P-selectin expressed on activated platelets causes formation of PNC. Moreover, platelets and platelet-derived P-selectin play an important role in thrombus growth at the site of atherosclerosis.[2] *In vivo* and *in vitro* studies have shown that shear stress and exposure to atherogenic stimuli, such as oxidization by low-density lipoprotein or cigarette smoking, induce rapid P-selectin-dependent aggregation and accumulation of leukocytes and platelets.[4,5,11] Activated platelets accumulating in thrombi at the site of ruptured atherosclerotic plaques will express CD62P. In clinical studies, P-selectin has been shown to be a marker of platelet activation related to adverse cardiovascular events such as hypertension, coronary artery disease, cerebrovascular disease, and peripheral arterial disease,[6,7,10–12] and also to be a predictor of cardiovascular events.[8,12] PNC, forming as a result of the interaction of platelet P-selectin and neutrophils also promotes platelet activation.[24] This is the first study to demonstrate that P-selectin and PNC were significantly correlated with arterial stiffness evaluated by PWV in normal subjects. In an analysis of four randomized trials, Hebert *et al*[29] showed that aspirin therapy was beneficial in the primary prevention of vascular disease. Higher levels of other membrane markers such as von Willebrand factor receptor are observed in activated platelets, which are affected by aspirin

or ticlopidine.[30] Therefore, our results indicate that, in the normal population, antiplatelet agents may play a role in preventing cardiovascular events through factors other than P-selectin.

Although the exact mechanism accounting for the relationship between platelet activation and arterial stiffness is unknown, it is possible to make the following speculations. When arterial stiffness is raised, shear stress might play an important role in platelet activation. Using cone-plate viscometry,[3] Goto *et al* showed that platelet activation (measured by P-selectin surface expression, von Willebrand factor-mediated platelet aggregation and translocation of GP Ibα) was induced by high shear stress of 10 800/s. Higher arterial stiffness increases blood flow velocity and produces a steep systolic pressure waveform,[31] and it is possible that the resulting increased shear stress could promote platelet activation. Another possible mechanism is that endothelial dysfunction may interact with arterial stiffness and platelet hyperactivity. Kobayashi *et al*[32] showed significant correlation between endothelial dysfunction measured by flow-mediated dilatation and ba-PWV. Platelets are also activated by endothelial dysfunction. On the other hand, activated platelets themselves may cause arterial stiffness via vascular smooth muscle cell growth factors and extracellular matrix modulator released from platelets, that is, PDGF.[33] However, this response also occurs at the site of endothelial injury. Further study is therefore required to clarify whether arterial stiffness causes platelet activation or alternatively whether platelet activation might result in arterial stiffness.

Limitations

Despite the small sample size, it is possible that the broad age range (23–77 years) of our subjects caused outliers in PWV and platelet activation. However, significant correlations were found when age and blood pressure were adjusted for, suggesting that the influence of age did not entirely explain the correlation between PWV and platelet activation. In the present study, baPWV was 14.1±3.0 m/s in men and 13.6±3.1 m/s in women; values higher than those reported by Yamashina *et al*.[20] Furthermore, it is not known whether such a relationship between arterial stiffness and platelet activation is found in patients with conditions such as hypertension, diabetes mellitus, coronary heart disease, and stroke. Further studies should be therefore performed in such patients, using larger sample sizes.

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References

1 Tenaglia AN *et al*. Levels of expression of P-selectin, E-selectin, and intercellular adhesion molecule-1 in coronary atherectomy specimens from patients with stable and unstable angina pectoris. *Am J Cardiol* 1997; 79: 742–747.
2 Hagberg IA, Roald HE, Lyberg T. Platelet activation in flowing blood passing growing arterial thrombi. *Arterioscler Thromb Vasc Biol* 1997; 17: 1331–1336.
3 Goto S *et al*. Effects of ticlopidine on von Willebrand factor-mediated shear-induced platelet activation and aggregation. *Platelets* 2001; 12: 406–414.
4 Davi G *et al*. Increased levels of soluble P-selectin in hypercholesterolemic patients. *Circulation* 1998; 97: 953–957.
5 Pernerstorfer T *et al*. Low-dose aspirin does not lower *in vivo* platelet activation in healthy smokers. *Br J Haematol* 1998; 102: 1229–1231.
6 Spencer CG *et al*. Von Willebrand factor, soluble P-selectin, and target organ damage in hypertension: a substudy of the Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT). *Hypertension* 2002; 40: 61–66.
7 Blann AD, Dobrotova M, Kubisz P, McCollum CN. von Willebrand factor, soluble P-selectin, tissue plasminogen activator and plasminogen activator inhibitor in atherosclerosis. *Thromb Haemost* 1995; 74: 626–630.
8 Blann AD, Faragher EB, McCollum CN. Increased soluble P-selectin following myocardial infarction: a new marker for the progression of atherosclerosis. *Blood Coagul Fibrinolysis* 1997; 8: 383–390.
9 Grau AJ *et al*. Increased fraction of circulating activated platelets in acute and previous cerebrovascular ischemia. *Thromb Haemost* 1998; 80: 298–301.
10 Serebruany VL *et al*. Uniform platelet activation exists before coronary stent implantation despite aspirin therapy. *Am Heart J* 2001; 142: 611–616.
11 Parker III C, Vita JA, Freedman JE. Soluble adhesion molecules and unstable coronary artery disease. *Atherosclerosis* 2001; 156: 417–424.
12 Lip GY *et al*. Sequential alterations in haemorheology, endothelial dysfunction, platelet activation and thrombogenesis in relation to prognosis following acute stroke: The West Birmingham Stroke Project. *Blood Coagul Fibrinolysis* 2002; 13: 339–347.
13 De Cesaris R, Ranieri G, Filitti V, Andriani A. Large artery compliance in essential hypertension. Effects of calcium antagonism and beta-blocking. *Am J Hypertens* 1992; 5: 624–628.
14 Asmar R *et al*. Assessment of arterial distensibility by automatic pulse wave velocity measurement. Validation and clinical application studies. *Hypertension* 1995; 26: 485–490.
15 Lehmann ED, Riley WA, Clarkson P, Gosling RG. Noninvasive assessment of cardiovascular disease in diabetes mellitus. *Lancet* 1997; 350Suppl 1: SI14–SI19.
16 Asmar J *et al*. Arterial stiffness and cardiovascular risk factors in a population-based study. *J Hypertens* 2001; 19: 381–387.
17 Mourad JJ *et al*. Creatinine clearance, pulse wave velocity, carotid compliance and essential hypertension. *Kidney Int* 2001; 59: 1334–1341.
18 Yamashina A *et al*. Validity, reproducibility, and clinical significance of noninvasive brachial-ankle pulse wave velocity measurement. *Hypertens Res* 2002; 25: 359–364.

19 Munakata M, Ito N, Nunokawa T, Yoshinaga K. Utility of automated brachial ankle pulse wave velocity measurements in hypertensive patients. *Am J Hypertens* 2003; 16: 653–657.
20 Yamashina A *et al.* Nomogram of the relation of brachial–ankle pulse wave velocity with blood pressure. *Hypertens Res* 2003; 26: 801–806.
21 Yamashina A *et al.* Brachial–ankle pulse wave velocity as a marker of atherosclerotic vascular damage and cardiovascular risk. *Hypertens Res* 2003; 26: 615–622.
22 Asmar R *et al.* Pulse pressure and aortic pulse wave are markers of cardiovascular risk in hypertensive populations. *Am J Hypertens* 2001; 14: 91–97.
23 Guerin AP *et al.* Impact of aortic stiffness attenuation on survival of patients in end-stage renal failure. *Circulation* 2001; 103: 987–992.
24 Peters MJ, Heyderman RS, Hatch DJ, Klein NJ. Investigation of platelet–neutrophil interactions in whole blood by flow cytometry. *J Immunol Methods* 1997; 209: 125–135.
25 Hirai T, Sasayama S, Kawasaki T, Yagi S. Stiffness of systemic arteries in patients with myocardial infarction. A noninvasive method to predict severity of coronary atherosclerosis. *Circulation* 1989; 80: 78–86.
26 Popele NM *et al.* Association between arterial stiffness and atherosclerosis: the Rotterdam Study. *Stroke* 2001; 32: 454–460.
27 Blann AD, Lip GY, Beevers DG, McCollum CN. Soluble P-selectin in atherosclerosis: a comparison with endothelial cell and platelet markers. *Thromb Haemost* 1997; 77: 1077–1080.
28 Bonfanti R, Furie BC, Furie B, Wagner DD. PADGEM (GMP140) is a component of Weibel-Palade bodies of human endothelial cells. *Blood* 1989; 73: 1109–1112.
29 Hebert PR, Hennekens CH. An overview of the 4 randomized trials of aspirin therapy in the primary prevention of vascular disease. *Arch Intern Med* 2000; 160: 3123–3127.
30 Goto S *et al.* Effects of ticlopidine on von Willebrand factor-mediated shear-induced platelet activation and aggregation. *Platelets* 2001; 12: 406–414.
31 Nichols WW, O'Rourke MF. Effect of age and of hypertension on wave travel and reflections. *Arterial Vasodilation: Mechanisms and Therapy.* Arnold: London, 1993.
32 Kobayashi K *et al.* Interrelationship between noninvasive measurements of atherosclerosis: flow-mediated dilation of brachial artery, carotid intima-media thickness and pulse wave velocity. *Atherosclerosis* 2004; 173: 13–18.
33 Dzau VJ, Gibbons GH. Vascular remodeling: mechanisms and implications. *J Cardiovasc Pharmacol* 1993; 21: S1–S5.

Myocardial interstitial choline and glutamate levels during acute myocardial ischaemia and local ouabain administration

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Abstract

Aim: Norepinephrine uptake transporters are known to reverse their action during acute myocardial ischaemia and to contribute to ischaemia-induced myocardial interstitial norepinephrine release. By contrast, functional roles of choline and glutamate transporters during acute myocardial ischaemia remain to be investigated. Because both transporters are driven by the normal Na^+ gradient across the plasma membrane in a similar manner to norepinephrine transporters, the loss of Na^+ gradient would affect the transporter function, which would in turn alter myocardial interstitial choline and glutamate levels. The aim of the present study was to examine the effects of acute myocardial ischaemia and the inhibition of Na^+-K^+ ATPase on myocardial interstitial glutamate and choline levels. **Methods:** In anaesthetized cats, we measured myocardial interstitial glutamate and choline levels while inducing acute myocardial ischaemia or inhibiting Na^+-K^+ ATPase by local administration of ouabain. **Results:** The choline level was not changed significantly by ischaemia (from 0.93±0.06 to 0.82±0.13 μM, mean±SE, n=6) and was decreased slightly by ouabain (from 1.30±0.06 to 1.05±0.07 μM, $P<0.05$, n=6). The glutamate level was significantly increased from 9.5±1.9 to 34.7±6.1 μM by ischaemia ($P<0.01$, n=6) and from 8.9±1.0 to 15.9±2.3 μM by ouabain ($P<0.05$, n=6). Inhibition of glutamate transport by trans-L-pyrrolidine-2,4-dicarboxylate (t-PDC) suppressed ischaemia- and ouabain-induced glutamate release. **Conclusion:** Myocardial interstitial choline level was not increased by acute myocardial ischaemia or by Na^+-K^+ ATPase inhibition. By contrast, myocardial interstitial glutamate level was increased by both interventions. The glutamate transporter contributed to glutamate release via retrograde transport.

Keywords

acetylcholine

cardiac microdialysis

cats

coronary artery occlusion

myocardium

norepinephrine

plasma transporters

vagus

Introduction

Acute myocardial ischaemia causes oxygen depletion and loss of ATP in the ischaemic region (Hearse 1979). Blockade of H^+-ATPase leads to norepinephrine (NE) leakage from storage vesicles and axoplasmic NE accumulation (Schömig *et al.* 1988). Intracellular acidosis causes Na^+ influx via Na^+/H^+ exchange. Inhibition of Na^+-K^+ ATPase activity reduces the Na^+ gradient across the plasma membrane. Because NE uptake transporters are driven by the normal Na^+ electrochemical gradient across the plasma membrane, axoplasmic NE accumulation and reduction of the Na^+ gradient cause reverse transport of NE from the intracellular space to the extracellular space (Schwartz 2000). Acute myocardial ischaemia evokes the myocardial interstitial NE release in the ischaemic region via retrograde NE transport, independently of efferent sympathetic nerve activity (Akiyama & Yamazaki 1999, Kawada *et al.* 2001, Schömig *et al.* 1984, Yamazaki *et al.* 1996).

Similarly to NE, choline and glutamate are taken up into cells by plasma membrane transporters driven by the Na^+ gradient (Schwartz 2000). We hypothesized that the loss of Na^+ gradient under ischaemic conditions would interfere with the transporter function, which would in turn alter myocardial interstitial choline and glutamate levels. Choline release has been suggested as an index of ischaemic degradation of the myocardial phospholipid bilayer in isolated, Tyrode-solution perfused rat hearts (Brühl *et al.* 2004). Glutamate can be a preferred myocardial fuel during ischaemia and may have protective effects on ischaemic myocardium (Arsenian 1998). Measuring myocardial interstitial levels of these molecules *in vivo* would contribute to understanding the pathophysiology of acute myocardial ischaemia. To test the hypothesis, we employed an *in vivo* cardiac microdialysis technique and measured myocardial interstitial choline and glutamate levels in anaesthetized cats (Akiyama *et al.* 1991, Akiyama *et al.* 1994, Kawada *et al.* 2001, Yamazaki *et al.* 1997). Acute myocardial ischaemia inevitably affects systemic haemodynamics and perfusion of the heart. To minimize such heamodynamic effects, we also examined the effects of Na^+-K^+ ATPase inhibition on the myocardial interstitial choline and glutamate levels by locally administering ouabain through a dialysis probe (Kawada *et al.* 2002, Yamazaki *et al.* 1999). The results of the present study indicated that the myocardial interstitial choline level was not increased by acute myocardial ischaemia or by Na^+-K^+ ATPase inhibition. By contrast, the myocardial interstitial glutamate level was increased by both interventions. The glutamate transporter contributed to glutamate release via retrograde transport.

Materials and Methods

Surgical Preparation

Animal care was conducted in strict accordance with the *Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences* approved by the Physiological Society of Japan. Adult cats weighing 2.0 to 4.8 kg were anaesthetized via an intraperitoneal injection of pentobarbital sodium (30-35 $mg \cdot kg^{-1}$) and ventilated mechanically with room air mixed with oxygen.

The depth of anaesthesia was maintained with a continuous intravenous infusion of pentobarbital sodium (1-2 $mg \cdot kg^{-1} \cdot h^{-1}$) through a catheter inserted via the right femoral vein. Mean systemic arterial pressure was monitored from a catheter inserted via the right femoral artery.

With the animal in the lateral position, the left fifth and sixth ribs were resected to expose the heart. When a coronary occlusion was necessary, a 3-0 silk suture was prepared around the left anterior descending coronary artery (LAD) just distal to the first diagonal branch. With a fine guiding needle, a dialysis probe was implanted into the left ventricular free wall perfused by the LAD. Heparin sodium (100 $U \cdot kg^{-1}$ bolus injection followed by a maintenance dose of 50 $U \cdot kg^{-1} \cdot h^{-1}$) was administered intravenously to prevent blood coagulation. At the end of the experiment the experimental animals were killed by an overdose of pentobarbital sodium. We confirmed that the dialysis probe had been implanted within the left ventricular myocardium.

Dialysis Technique

We designed a transverse dialysis probe (Akiyama *et al.* 1991, Akiyama *et al.* 1994). For measurements of small molecular compounds including ACh, choline, and glutamate, we used a dialysis fiber of 50,000 molecular weight cutoff (13 mm length, 310 μm O.D., 200 μm I.D.; PAN-1200, Asahi Chemical, Japan) with both ends glued to polyethylene tubes (20 cm length, 500 μm O.D., 200 μm I.D.). The dialysis probe was perfused at a rate of 2 $\mu l \cdot min^{-1}$ with Ringer solution. Each sample was collected in a microtube containing 3 μl of phosphate buffer (100 mM, pH 3.5). A cholinesterase inhibitor eserine (100 μM) was added to the perfusate to measure ACh. A preliminary examination indicated that whether the perfusate contained eserine did not affect myocardial interstitial choline levels significantly. Dead space volume between the dialysis fiber and the sample microtube was identical for ACh, choline, and glutamate measurements, and the sampling was performed taking into account the time for dialysate to traverse the dead space volume.

The dialysate ACh and choline levels were measured directly by high performance liquid chromatography with electrochemical detection. The absolute detection limits of ACh and choline, determined with a signal-to-noise ratio of 3, were 10 and 5 fmol per injection, respectively. The dialysate glutamate level was measured by kinetic enzymatic analysis with CMA 600. The absolute detection limit of glutamate was 1 μM per injection.

Protocols

All protocols were started from 2 h after implanting the dialysis probe. To examine changes in myocardial interstitial ACh and choline levels during acute myocardial ischaemia (n = 6), after collecting a 15-min baseline dialysate sample, we occluded the LAD for 60 min and obtained four consecutive 15-min dialysate samples. The full-length of the implanted dialysis fiber was located within the ischaemic area judged by discolouration of myocardium during the LAD occlusion. We then released the occlusion and collected a 15-min dialysate sample during reperfusion. To examine changes in myocardial ACh and choline levels in response to local ouabain administration (n = 6),

after collecting a 15-min baseline dialysate sample, we replaced the perfusate with Ringer solution containing 100 μM ouabain and collected four consecutive 15-min dialysate samples.

In different groups of animals, myocardial interstitial glutamate levels were measured during acute myocardial ischaemia (n = 6) and during local administration of ouabain (n = 6). To elucidate the role of the glutamate transporter, we also examined the effects of glutamate transport inhibition by trans-L-pyrrolidine-2,4-dicarboxylate (t-PDC, 10 mM) on myocardial interstitial glutamate levels during acute myocardial ischaemia (n = 7) and local administration of ouabain (n = 7). t-PDC was locally administered through the dialysis probe to avoid systemic effects.

Statistical Analysis

All data are presented as mean ± SE values. In each protocol, the effects of myocardial ischaemia or local ouabain administration were examined using one-way analysis of variance followed by Dunnett's test against the corresponding baseline level (Glantz 2002). The baseline as well as maximum glutamate levels with and without glutamate transport inhibition were compared by an unpaired-t test during acute myocardial ischaemia or during local ouabain administration (Glantz 2002). Differences were considered to be significant when $P<0.05$.

Results

Figure 1A shows myocardial interstitial ACh level during acute myocardial ischaemia. The ACh level was increased by LAD occlusion, becoming approximately 15 times higher than the baseline level at 30-45 and 45-60 min of ischaemia. The ACh level decreased toward the baseline level upon reperfusion. Figure 1B illustrates myocardial interstitial choline level during acute myocardial ischaemia. The choline level did not change significantly throughout the ischaemic and reperfusion periods.

Figure 2A shows changes in myocardial interstitial ACh level during local administration of ouabain. The ACh level was increased by the inhibition of Na^+-K^+ ATPase, becoming approximately 9 times higher than the baseline level at 15-30 min. The ACh level then decreased but remained significantly higher than the baseline level. Figure 2B illustrates the myocardial interstitial choline level during local administration of ouabain. The choline level was significantly lower at 0-15 and 45-60 min when compared with the baseline level.

Figure 3A shows changes in myocardial interstitial glutamate level during acute myocardial ischaemia. LAD occlusion increased the glutamate level to approximately 3.5 times higher than the baseline level at 0-15 min. Thereafter, the glutamate level was significantly higher than the baseline level throughout the ischaemic and reperfusion periods. Figure 3B illustrates the effects of glutamate transport inhibition on the ischaemia-induced glutamate release. The baseline glutamate level was significantly decreased by glutamate transport inhibition ($P<0.05$). Although acute myocardial ischaemia and reperfusion significantly increased the glutamate level relative to the baseline level,

the maximum glutamate level was attenuated to approximately one-fifth compared with that observed without glutamate transport inhibition ($P<0.05$).

Figure 4A shows changes in myocardial interstitial glutamate level during the local administration of ouabain. Ouabain administration did not change the glutamate level at 0-15 min but increased the glutamate level thereafter. The glutamate level became approximately 1.8 times higher than the baseline level at 30-45 min. Figure 4B illustrates the effects of glutamate transport inhibition on ouabain-induced glutamate release. The baseline glutamate level was significantly decreased by the inhibition of glutamate transport ($P<0.05$). Although ouabain administration increased the glutamate level relative to the baseline level, the maximum glutamate level was suppressed to approximately one-third of that observed without glutamate transport inhibition ($P<0.05$).

Discussion

We have shown that acute myocardial ischaemia and local inhibition of Na^+-K^+ ATPase increased myocardial interstitial glutamate level but not choline level. Despite the similar Na^+ gradient dependency of corresponding transporters, myocardial interstitial glutamate and choline levels showed differential responses to the two interventions.

Changes in myocardial interstitial choline level

In the vagal nerve endings, ACh is hydrolyzed to acetate and choline by acetylcholinesterase (Nicholls 1994). Choline is then taken up into the vagal nerve endings by the choline transporter driven by the Na^+ gradient. We hypothesized that loss of Na^+ gradient during acute myocardial ischaemia or local ouabain administration would increase the myocardial interstitial choline level by the interruption of choline uptake. Contrary to our hypothesis, acute myocardial ischaemia did not change myocardial interstitial choline level in the ischaemic region (Fig. 1B). Ouabain administration decreased the myocardial interstitial choline level at 0-15 and 45-60 min (Fig. 2B).

Possible explanations for the absence of ischaemia- or ouabain-induced choline release are as follows. First, choline uptake is the rate-limiting step for ACh synthesis (Lockman & Allen 2002). Because choline in the intracellular space is rapidly consumed for ACh synthesis, the axoplasmic choline concentration might have been too low to evoke reverse transport by the choline transporter. Second, plasma choline concentration is stabilized by *de novo* choline synthesis from the catabolism of phosphatidylcholine found in cell membranes (Lockman & Allen 2002). Potential choline release may have been counterbalanced by the local stabilization mechanisms. Taking into account the recovery rate of the dialysis probe (approximately 30%), the myocardial interstitial choline concentration was 3-5 μM. Although the estimated concentration was lower than the highly regulated plasma choline concentration of approximately 10 μM, it was much higher than the ischaemia-induced maximum choline release (approximately 0.6 μM) in isolated rat hearts reported by Brühl *et al.* (2004). The present results suggests that myocardial interstitial choline level may not serve as such an indicator of myocardial ischaemia in blood-perfused *in vivo* feline hearts.

By contrast with myocardial interstitial choline level, myocardial interstitial ACh level was increased both by acute myocardial ischaemia and by local administration of ouabain. Because ischaemia-induced ACh release was observed after vagal nerve transection in a previous study (Kawada *et al.* 2000), a Ca^{2+} channel independent, regional release mechanism appears to be involved. Several reports have suggested that ouabain or ischaemia-induced intracellular Na^+ accumulation could elevate intracellular Ca^{2+} level via Na^+/Ca^{2+} exchange (Li *et al.* 2000, Mochizuki & Jiang 1998). The elevation of intracellular Ca^{2+} level may be associated with ACh release. Our previous study indicated that intracellular Ca^{2+} overload due to Ca^{2+} mobilization is responsible for the ACh release evoked by ischaemia (Kawada *et al.* 2000).

Changes in myocardial interstitial glutamate levels

Although the glutamate transporter family differs from the NE transporter family in that it requires countertransport of K^+ instead of cotransport of Cl^-, its primary driving force is the Na^+ gradient across the plasma membrane (Schwartz 2000). Therefore, interventions that reduce the plasma Na^+ gradient are likely to cause reverse transport of glutamate, in a similar manner to the reverse transport of NE. Acute myocardial ischaemia increased the myocardial interstitial glutamate level (Fig. 3A) as consistent with previous reports (Bäckström *et al.* 2003, 11. Kennergren *et al.* 1997, Kennergren *et al.* 1999, Song *et al.* 1996). Inhibition of Na^+-K^+ ATPase also induced myocardial interstitial glutamate release (Fig. 4A). Glutamate release during acute myocardial ischaemia and local ouabain administration was significantly attenuated by the inhibition of glutamate transport (Figs. 3B and 4B), suggesting the involvement of reverse transport by the glutamate transporter. Glutamate plays a vital role in keeping nitrogen balance in cells as a common amino acid in transamination reactions. The high intra-to-extracellular concentration ratio of glutamate would contribute to the retrograde transport by the glutamate transporter during the loss of normal Na^+ gradient.

In the case of myocardial interstitial NE levels, local blockade of NE uptake increased baseline NE levels, suggesting the accumulation of NE spontaneously released into the synaptic cleft (Akiyama & Yamazaki 1999). We therefore predicted that the inhibition of glutamate transport would increase the baseline glutamate level. However, the inhibition of glutamate transport actually decreased the baseline glutamate level (Figs. 3 and 4), suggesting that spontaneous glutamate release rather than glutamate uptake had occurred under baseline conditions. The insertion of a dialysis probe inevitably damages the myocardium. Although we waited for 2 h after implantation of the dialysis probe and the glutamate level declined with time, glutamate release from damaged myocardium may have continued. Notwithstanding this limitation, we were able to detect glutamate release in response to acute myocardial ischaemia and inhibition of Na^+-K^+ ATPase. Therefore, our interpretation that glutamate release was dependent on the reverse transport of glutamate transporter may be reasonable.

Supplementing the heart with glutamate has been shown to have beneficial effect on the recovery of contractile function in post-surgical patients (Arsenian 1998). The myocardial interstitial glutamate level remained increased during 15-min reperfusion whereas the myocardial interstitial ACh level returned toward the baseline level. Although the reason for different responses upon reperfusion was unanswered in the present study, the sustained increase in the glutamate level may have therapeutic effect on its own.

In conclusion, acute myocardial ischaemia and inhibition of Na^+-K^+ ATPase did not increase myocardial interstitial choline level despite a significant increase in myocardial interstitial ACh level. By contrast, both interventions significantly increased the myocardial interstitial glutamate level. The glutamate transporter contributed to myocardial interstitial glutamate release via retrograde transport.

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References

1. Akiyama, T. & Yamazaki, T. 1999. Norepinephrine release from cardiac sympathetic nerve endings in the in vivo ischemic region. *J Cardiovasc Pharmacol* **34** (Suppl 4), S11-S14.
2. Akiyama, T., Yamazaki, T. & Ninomiya, I. 1991. In vivo monitoring of myocardial interstitial norepinephrine by dialysis technique. *Am J Physiol Heart Circ Physiol* **261**, H1643-H1647.
3. Akiyama, T., Yamazaki, T. & Ninomiya, I. 1994. In vivo detection of endogenous acetylcholine release in cat ventricles. *Am J Physiol Heart Circ Physiol* **266**, H854-H860.
4. Arsenian M. 1998. Potential cardiovascular applications of glutamate, aspartate, and other amino acids. *Clin Cardiol* **21**, 620-624.
5. Bäckström, T., Goiny, M., Lockowandt, U., Liska, J. & Franco-Cereceda, A. 2003. Cardiac outflow of amino acids and purines during myocardial ischemia and reperfusion. *J Appl Physiol* **94**, 1122-1128.
6. Brühl, A., Hafner, G. & Löffelholz, K. 2004. Release of choline in the isolated heart, an indicator of ischemic phospholipid degradation and its protection by ischemic preconditioning: No evidence for a role of phospholipase D. *Life Sci* **75**, 1609-1620.
7. Glantz, SA. 2002. Primer of Biostatistics 5th ed. McGraw-Hill, New York.
8. Hearse, D. J. 1979. Oxygen deprivation and early myocardial contractile failure: a reassessment of the possible role of adenosine triphosphate. *Am J Cardiol* **44**, 1115-1121.

9. Kawada, T., Yamazaki, T., Akiyama, T. *et al.* 2000. Differential acetylcholine release mechanisms in the ischemic and non-ischemic myocardium. *J Mol Cell Cardiol* **32**, 405-414.
10. Kawada, T., Yamazaki, T., Akiyama, T. *et al.* 2001. Vagosympathetic interactions in ischemia-induced myocardial norepinephrine and acetylcholine relase. *Am J Physiol Heart Circ Physiol* **280**, H216-H221.
11. Kawada, T., Yamazaki, T., Akiyama, T. *et al.* 2001. In vivo assessment of acetylcholine-releasing function at cardiac vagal nerve terminals. *Am J Physiol Heart Circ Physiol* **281**, H139-H145.
12. Kawada, T., Yamazaki, T., Akiyama, T. *et al.* 2002. Disruption of vagal efferent axon and nerve terminal function in the postischemic myocardium. *Am J Physiol Heart Circ Physiol* **283**, H2687-H2691.
13. Kennergren, C., Mantovani, V., Lönnroth, P., Nyström, B., Berglin, E. & Hamberger, A. 1999. Extracellular amino acids as markers of myocardial ischemia during cardioplegic heart arrest. *Cardiology* **91**, 31-40.
14. Kennergren, C., Nyström, B., Nyström, U. *et al.* 1997. In situ detection of myocardial infarction in pig by measurements of aspartate aminotransferase (ASAT) activity in the interstitial fluid. *Scand Cardiovasc J* **31**, 343-349.
15. Li, S., Jiang, Q., Stys, P. K. 2000. Important role of reverse Na^{+}-Ca^{2+} exchange in spinal cord white matter injury at physiological temperature. *J Neurophysiol* **84**, 1116-1119.
16. Lockman, P. R. & Allen, D. D. 2002. The transport of choline. *Drug Dev Ind Pharm* **28**, 749-771.
17. Mochizuki, S. & Jiang, C. 1998. Na^{+}/Ca^{++} exchanger and myocardial ischemia/reperfusion. *Jpn Heart J* **39**, 707-714.
18. Nicholls, D. G. 1994. Acetylcholine. In: Proteins, transmitters and synapses. pp. 186-199. Blackwell Science, London.
19. Schömig, A., Dart, A. M., Dietz, R., Mayer, E. & Kübler, W. 1984. Release of endogenous catecholamines in the ischemic myocardium of the rat. Part A: Locally mediated release. *Circ Res* **55**, 689-701.
20. Schömig, A., Kurz, T., Richardt, G. & Schömig, E. 1988. Neuronal sodium homeostasis and axoplasmic amine concentration determine calcium-independent noradrenaline release in normoxic and ischemic rat heart. *Circ Res* **63**, 214-226.
21. Schwartz, J. H. 2000. Neurotransmitters. In: Kandel, E. R., Schwartz, J. H. & Jessell, T. M. (eds) Principles of Neural Science 4th ed. pp. 280-297, McGraw-Hill. New York.
22. Song, D., O'Regan, M. H. & Phillis, J. W. 1996. Release of the excitotoxic amino acids, glutamate and aspartate, from the isolated ischemic/anoxic rat heart. *Neurosci Lett* **220**, 1-4.
23. Yamazaki, T., Akiyama, T. & Kawada, T. 1999. Effects of ouabain on in situ cardiac sympathetic nerve endings. *Neurochem Int* **35**, 439-445.

24. Yamazaki, T., Akiyama, T., Kitagawa, H., Takauchi, Y. & Kawada, T. 1996. Elevation of either axoplasmic norepinephrine or sodium level induced release of norepinephrine from cardiac sympathetic nerve terminals. *Brain Res* **737**, 343-346.
25. Yamazaki, T., Akiyama, T., Kitagawa, H., Takauchi, Y., Kawada, T. & Sunagawa, K. 1997. A new, concise dialysis approach to assessment of cardiac sympathetic nerve terminal abnormalities. *Am J Physiol Heart Circ Physiol* **272**, H1182-H1187.

Legends to figures

Figure 1

Changes in myocardial interstitial acetylcholine (ACh) level (A) and choline level (B) during coronary artery occlusion and reperfusion. Myocardial interstitial ACh level was significantly increased by acute myocardial ischaemia, while myocardial interstitial choline level was not changed. Data are means±SE. $^{\dagger}P<0.01$ from baseline.

Figure 2

Changes in myocardial interstitial acetylcholine (ACh) level (A) and choline level (B) in response to the local administration of ouabain. Myocardial interstitial ACh level was significantly increased by ouabain. In contrast, myocardial interstitial choline level was decreased by ouabain. Data are means±SE. $^{\dagger}P<0.01$ from baseline.

Figure 3

Changes in myocardial interstitial glutamate level during coronary artery occlusion and reperfusion without (A) and with (B) the inhibition of glutamate transporter. The glutamate level was significantly increased by acute myocardial ischaemia. The ischaemia-induced glutamate release was suppressed by the inhibition of glutamate transporter. Data are means±SE. $^{\dagger}P<0.01$ and $^{*}P<0.05$ from baseline.

Figure 4

Changes in myocardial interstitial glutamate level in response to the local administration of ouabain without (A) and with (B) the inhibition of glutamate transporter. The glutamate level was significantly increased by ouabain administration. The ouabain-induced glutamate release was suppressed by the inhibition of glutamate transporter. Data are means±SE. $^{\dagger}P<0.01$ from baseline.