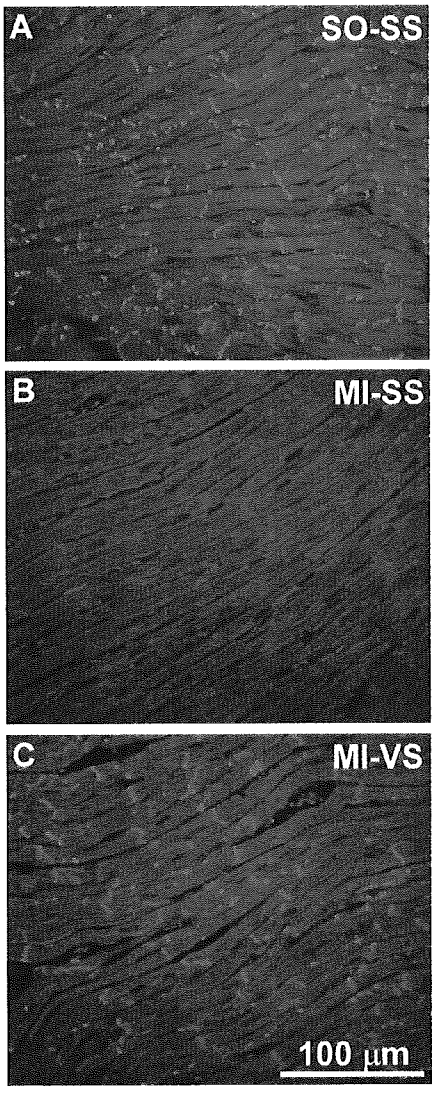

Figure 4. Representative confocal images of rat left ventricles from SO-SS rats (A), MI-SS rats (B), and MI-VS rats (C). Positive immunoreactive signals were concentrated in discrete spots at sites of intercellular apposition (red).

inhibits loss of the phosphorylated isoform of Cx43 during acute MI. Although the precise mechanism by which VS modulates the dephosphorylation of Cx43 remains unknown, it is most likely that VS exerts its antiarrhythmogenic effects on ischemic ventricular myocytes through the preserved function of Cx43.

VS and Antiarrhythmogenic Properties

VS has already been reported to prevent ventricular fibrillation in dogs.[14] In the present study, we hypothesized that VS exerts its antiarrhythmogenic properties by maintaining electrical coupling with ventricular cardiomyocytes as a result of prevention of Cx43 dephosphorylation induced by acute MI. However, because VS simultaneously evokes a bradycardiac effect, the question remains whether the heart rate deceleration caused by VS is a primary mechanism for antiarrhythmic properties during MI. In a preliminary study, we confirmed

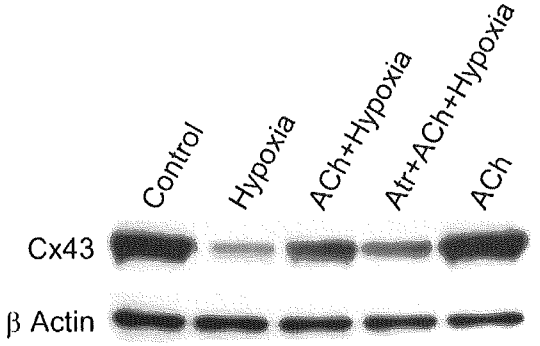

Figure 5. Representative immunoblots of homogenates from primary cultured rat cardiomyocytes probed with polyclonal anti-Cx43 antibody. Hypoxic treatment for 30 minutes decreased phosphorylated Cx43. ACh treatment prevented hypoxia-induced loss of phosphorylated Cx43. Atr inhibited ACh effect. Under normoxic conditions, ACh significantly increased phosphorylated isoform of Cx43 compared with control group.

that short-term exposure of cultured cardiomyocytes to ACh only before hypoxia prevented the hypoxia-induced loss of phosphorylated Cx43 (unpublished observations, 2004). Therefore, it is conceivable that ACh had a cardioprotective effect independent of the heart rate–slowing mechanism during hypoxia or ischemia. To further clarify such a preconditioning effect in in vivo experiments, we examined whether hearts preconditioned by VS were insusceptible to ischemia-induced VT and whether pcVS prevented the ischemia-induced loss of Cx43. As expected, we confirmed that pcVS exerted its antiarrhythmogenic effects and sustained the level of phosphorylated Cx43 during ischemia. These results suggest that VS or ACh had a cardioprotective effect independent of the heart rate–slowing mechanism.

It is well recognized that Cx43, which is the principal component of ventricular gap-junction proteins, contributes to intercellular communication and electrical coupling. Beardslee et al[21] showed that Cx43 underwent marked dephosphorylation during the process of electrical uncoupling induced by ischemia. Genetically engineered Cx43-deficient ($Cx43^{+/-}$ or $Cx43^{-/-}$) mice have been reported to be markedly susceptible to ischemia-induced VT.[12,13,22] In the present study, VS drastically reduced the incidence of VT and prevented the loss of phosphorylated Cx43 during acute MI. Therefore, functional preservation of Cx43 by VS would play an important role in antiarrhythmogenic properties during acute MI.

The result that ACh administration ameliorated the hypoxia-induced loss of dye coupling in cardiomyocytes is consistent with that of ACh-induced upregulation of phosphorylated Cx43. Under normoxic conditions, ACh did not slow down the spontaneous beating rate of cardiomyocytes. Hypoxia stopped the beating and diminished the phosphorylated isoform of Cx43; however, even under hypoxic conditions, ACh preserved the spontaneous beating and the phosphorylated isoform of Cx43. Therefore, it is conceivable that ACh has a cardioprotective effect independent of the beating rate.

Upregulation of Cx43 has been reported to accelerate spontaneous beating in cultured cardiomyocytes.[23] Moreover,

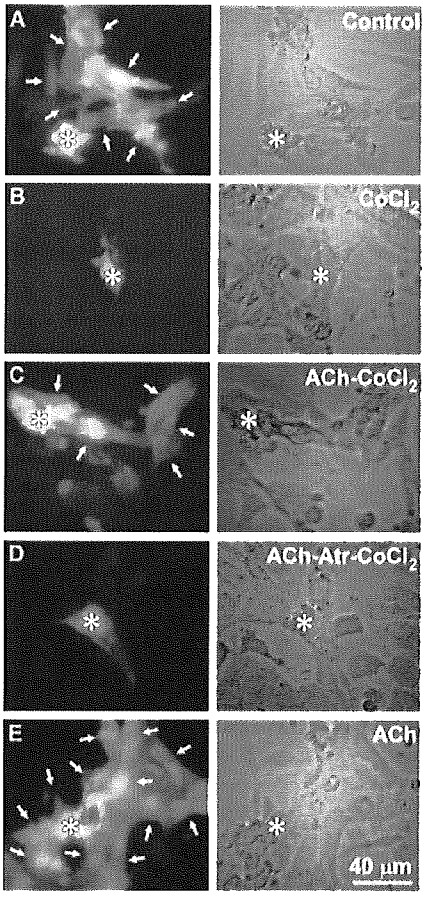

Figure 6. LY dye transfer assay in primary cultured rat cardiomyocytes. Each panel consists of fluorescent micrograph and transmission light micrograph. A, Control cultured cardiomyocytes. B, Cardiomyocytes treated with $CoCl_2$. C, Cardiomyocytes treated with ACh and $CoCl_2$. D, Cardiomyocytes treated with Atr, ACh, and $CoCl_2$. E, Cardiomyocytes treated with ACh alone. Asterisks indicate dye-injected cells; white arrows, coupled neighbors.

cultured cardiomyocytes from genetically engineered Cx43-deficient ($Cx43^{-/-}$) mice demonstrated slow spontaneous beating rates and were poorly synchronized with each other compared with wild-type cultured cardiomyocytes.[24] From these findings and the present results, we speculate that ACh exerts its antiarrhythmogenic properties on ischemic or hypoxic hearts by preserving Cx43 and that such a beneficial effect is independent of its bradycardiac effect.

The spatial distribution of Cx43 can influence electrical stability of the heart. A more recent study by Poelzing and Rosenbaum[11] has shown that the transmural derangement of Cx43 expression can potentially be an arrhythmogenic substrate in the canine model of pacing-induced heart failure. Although we did not evaluate the transmural heterogeneity of Cx43 expression in the present study, such an analysis would be needed to clarify precise mechanisms for the antiarrhythmogenic effects of VS.

How Does VS Modulate Phosphorylated Cx43?

Three potential mechanisms might be involved in the linkage between VS and the sustained phosphorylated-protein level of Cx43 during acute MI. First, VS may activate several protein kinases and induce phosphorylation of Cx43 through muscarinic receptors.[25] Liu et al[26] demonstrated that ACh prevented ischemic injury in cultured cardiomyocytes by activating protein kinase C, and the protective effect was mediated through a nitric oxide–dependent pathway. Second, VS can block the degradation pathway of Cx43 during acute MI. It has been reported that Cx43 is a short-lived protein with a half-life of only 1 to 3 hours in adult hearts[20] and that both lysosomal and proteasomal degradation play distinct roles in the life cycle of Cx43.[27] Our observations in the present study suggest that VS may prevent the ischemia-induced loss of Cx43 as a consequence of inhibition of its degradation pathway. Third, VS is also postulated to suppress excessive inflammation. Recently, Tracey and colleagues[28,29] identified a novel molecular link between the vagus nerve system and an antiinflammatory response to disease. They suggest that VS exerts an antiinflammatory effect via the nicotinic ACh receptor α7-subunit expressed in macrophages. The release of tumor necrosis factor-α from macrophages is inhibited by nicotinic stimulation. In contrast, the present results indicate that VS exerts its antiarrhythmogenic effects via muscarinic cholinergic receptors.

Study Limitations

In the present study, we did not measure the myocardial interstitial level of ACh at the ischemic region during VS. Therefore, it is unclear whether the cardiac vagal efferent fiber innervating the ischemic region can release its neurotransmitter in response to electrical stimulation. An in vivo microdialysis technique has enabled monitoring of the local concentration of neurotransmitters such as catecholamines, amino acids, and ACh. In the cat heart, Kawada et al showed that VS increased the myocardial interstitial ACh level even in the ischemic region (e-mail, February 12, 2004). Although their previous study[30] showed that acute MI induced the nerve-firing independent release of ACh from the vagal terminal, the electrical stimulation of the vagal efferent during acute MI produced the significant additive release of ACh to the myocardial interstitial space. Such an additional release of ACh in response to electrical stimulation during acute MI would play an important role in Cx43 preservation in the ischemic region.

Conclusion and Future Aspects

The present study demonstrates that VS exerts antiarrhythmogenic effects during acute MI accompanied by prevention of the loss of phosphorylated Cx43. The preserved function of Cx43 may improve electrical instability during acute MI. In view of the present results, we can provide an alternative therapeutic strategy with a neural interface approach. We have already developed the sympathetic interface approach for the treatment of central baroreflex failure in rats.[31] To establish the therapeutic strategy shown here, further studies are required.

Acknowledgments

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CLINICAL PERSPECTIVE

Increased cardiac vagal tone reduces ventricular arrhythmias during acute myocardial ischemia and has been linked to a lower risk of sudden arrhythmic death. Although the benefit of bradycardia associated with vagal tone is well appreciated, this may not be the only benefit. Cardiomyocytes are electrically coupled to one another through gap junctions. This coupling is critical to maintenance of cardiac electrical stability. Uncoupling occurs during ischemia and promotes heterogeneity of repolarization and slowing of conduction, with proarrhythmic effects. In the present study, short-term vagal stimulation (VS) was applied before or during acute ischemia in rats. VS protected against ventricular arrhythmias. Furthermore, VS preserved a phosphorylated form of connexin 43 (Cx43), a major subtype of gap-junction proteins in ventricles. In vitro studies of rat primary-cultured cardiomyocytes showed that ACh, a vagal efferent neurotransmitter, effectively prevented hypoxia-induced loss of phosphorylated Cx43 proteins and maintained cell-to-cell communication. Antiarrhythmic properties of VS and ACh were mediated via muscarinic receptors but were independent of heart rate deceleration. That cellular coupling can be improved through neural stimulation may lead to novel therapeutic strategies for preventing ventricular fibrillation during ischemia.

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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[1] 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 (3.13%). 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 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

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.
*$P<0.05$.
**$P<0.01$.

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 cardiovascular 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.

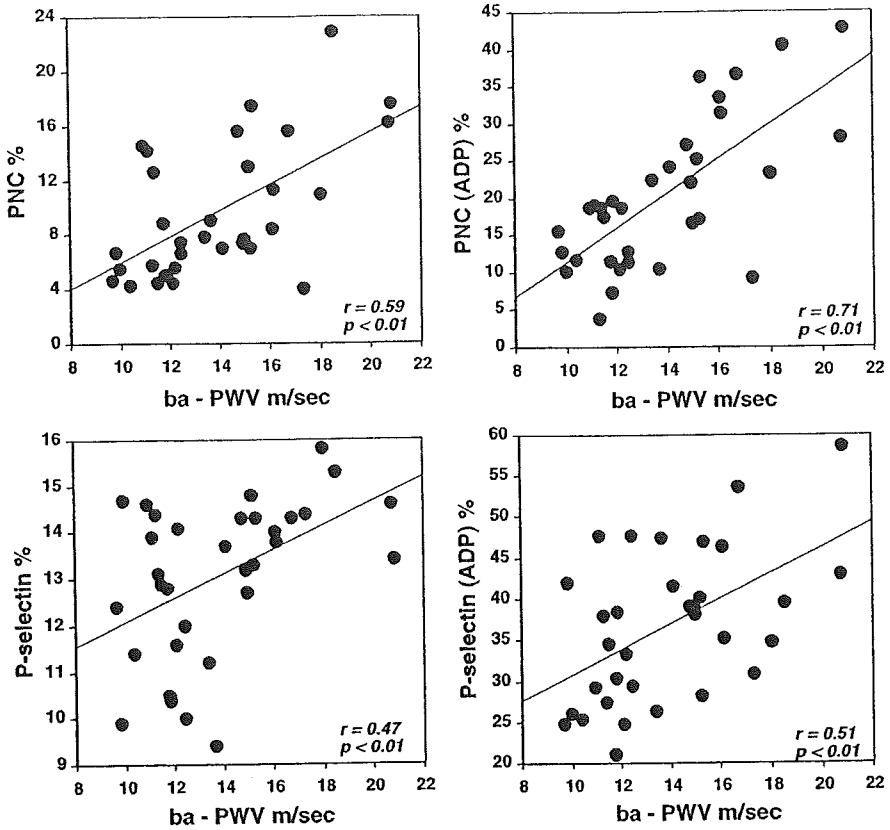

Figure 1 Correlation between ba-PWV and PNC (upper two panels). PNC = platelet neutrophil complexes; ADP = adenosine diphosphate; Δ-PNC = PNC (ADP)–PNC; Δ-P-selectin = P-selectin (ADP)–P-selectin; hb-PWV = heart–brachial pulse wave velocity; ba-PWV = brachial–ankle pulse wave velocity.

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. 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 rate of $10\,800\,s^{-1}$. 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, ba-PWV 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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Acetylcholine from vagal stimulation protects cardiomyocytes against ischemia and hypoxia involving additive non-hypoxic induction of HIF-1α

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Abstract Electrical stimulation of the vagal efferent nerve improves the survival of myocardial infarcted rats. However, the mechanism for this beneficial effect is unclear. We investigated the effect of acetylcholine (ACh) on hypoxia-inducible factor (HIF)-1α using rat cardiomyocytes under normoxia and hypoxia. ACh posttranslationally regulated HIF-1α and increased its protein level under normoxia. ACh increased Akt phosphorylation, and wortmannin or atropine blocked this effect. Hypoxia-induced caspase-3 activation and mitochondrial membrane potential collapse were prevented by ACh. Dominant-negative HIF-1α inhibited the cell protective effect of ACh. In acute myocardial ischemia, vagal nerve stimulation increased HIF-1α expression and reduced the infarct size. These results suggest that ACh and vagal stimulation protect cardiomyocytes through the PI3K/Akt/HIF-1α pathway.

Keywords: Acetylcholine; Ischemia; Apoptosis; Protein kinases

1. Introduction

The prognosis of patients with chronic heart failure remains poor, due to progressive remodeling of the heart and lethal arrhythmia. Acute ischemia or hypoxia causes loss of cardiomyocytes, followed by remodeling in the chronic phase. Although various therapeutic approaches have been introduced, including implantable defibrillators [1], a more effective modality of therapy has been anticipated for several years. A recent animal study by Li et al. [2] demonstrated that vagal nerve stimulation prevented ventricular remodeling after myocardial infarction, suggesting a novel therapeutic strategy against heart failure. Furthermore, Krieg et al. [3] reported that acetylcholine (ACh) has a cardioprotective effect. Although nitric oxide (NO) is supposed to be a major signaling molecule induced by ACh, a mechanism for the beneficial effect of vagal nerve stimulation on cardiomyocytes remains to be clarified. To investigate this mechanism, we hypothesized that vagal stimulation or ACh directly triggers a cell survival signal that is subsequently amplified and leads to protection of the cardiomyocytes from acute ischemic conditions, and that this effect of ACh, if continued, could be responsible for chronic cardioprotection.

In the present study, we focused on demonstrating the cellular action of ACh through hypoxia-inducible factor (HIF)-1α. HIF-1α is a transcription factor that is important for cell survival under hypoxia. HIF-1α activates the expression of many genes indispensable for cell survival [4,5]. Under normoxia, the HIF-1α protein level is very low, due to proteasomal degradation through with von Hippel–Lindau tumor suppressor protein (VHL). However, HIF-1α escapes from this degradation under hypoxia, and this is recognized as the hypoxic pathway [6,7]. Recently, it was revealed that HIF-1α can be also induced via a non-hypoxic pathway by angiotensin II [8,9]. Taken together, it is conceivable that HIF-1α induction is one of the adaptation processes to hypoxia and ischemia, and that additional induction of HIF-1α during ischemia via a non-hypoxic pathway could provide further cardioprotection.

Therefore, we investigated the direct effects of ACh on survival signaling in cardiomyocytes and of vagal stimulation on hearts. The results suggest that ACh and vagal stimulation protect cardiomyocytes from acute hypoxia and ischemia via additional HIF-1α protein induction through a non-hypoxic pathway.

2. Materials and methods

2.1. Cell culture

To examine the effect of ACh on cardiomyocytes, H9c2 cells as well as primary cardiomyocytes isolated from neonatal rats were used. H9c2 cells, which are frequently used to investigate signal transduction and channels in rat cardiomyocytes, are derived from rat embryonic ventricular cardiomyocytes. H9c2 cells were incubated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics. Primary cardiomyocytes were isolated from 2–3-day-old neonatal WKY rats and incubated in DMEM/Ham F-12 containing 10% FBS. HEK293 cells and HeLa cells cultured in DMEM containing 10% FBS were also used.

2.2. Western Blot analysis

H9c2 cells and primary cardiomyocytes were treated with 1 mM ACh to evaluate expression of HIF-1α protein under normoxia or with 1 mM *S*-nitroso-*N*-acetylpenicillamine (SNAP) to study the signal transduction. To investigate the signal transduction, H9c2 cells were pretreated with a PI3K inhibitor, (wortmannin; 300 nM), a muscarinic receptor, (atropine; 1 mM), a transcriptional inhibitor, (actinomycin D; 0.5 μg/ml) or a protein synthesis inhibitor, (cycloheximide; 10 μg/ml), followed by ACh treatment. Cell lysates were mixed with a sample

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buffer, fractionated by 10% SDS–PAGE and transferred onto membranes. The membranes were incubated with primary antibodies against HIF-1α (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Akt and phospho-Akt (Cell Signaling Technology, Beverly, MA, USA), and α-tubulin (Lab Vision, Fremont, CA, USA), and then reacted with an HRP-conjugated secondary antibody (BD Transduction Laboratories, San Diego, CA, USA). Positive signals were detected with an enhanced chemiluminescence system (Amersham, Piscataway, NJ, USA). In each study, the experiments were performed in duplicate and repeated 3–5 times ($n = 3$–5). Representative data are shown.

2.3. MTT activity assay

To evaluate the effects of hypoxia and ACh on the mitochondrial function of cardiomyocytes, we measured 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction activity in H9c2 or HEK293 cells under hypoxia (1% oxygen concentration), in the presence or absence of ACh. The cells were pretreated with 1 mM ACh for 12 h, and then subjected to hypoxia for 12 h. At 4 h before sampling, the MTT reagents were added to the culture and incubated.

2.4. Caspase-3 activity assay

Caspase-3 activity was measured using a CPP32/Caspase-3 Fluorometric Protease Assay Kit, (Chemicon International, Temecula, CA, USA). Hypoxia-treated H9c2 cells with or without 1 mM ACh pretreatment were lysed and the cytosolic extract was added to the caspase-3 substrate. A fluorometer equipped with a 400-nm excitation filter and 505-nm emission filter was used to measure the samples.

2.5. DePsipher assay

To examine the effects of hypoxia and ACh on the mitochondrial electrochemical gradient, we analyzed cardiomyocytes using a DePsipher™ Mitochondrial Potential Assay Kit (Trevigen, Gaithersburg, Maryland, USA). Apoptotic cells, which undergo mitochondrial membrane potential collapse cannot accumulate the DePsipher reagent in their mitochondria. As a result, apoptotic cells show decreased red fluorescence in their mitochondria, and the reagent remains in the cytoplasm as a green fluorescent monomer. Therefore, apoptotic cells were easily differentiated from healthy cells, which showed more red fluorescence.

2.6. Evaluation of NO production

NO production was measured using the 4,5-diaminofluoresceindiacetate (DAF-2DA; Alexis, Lausen, Switzerland) fluorometric NO detection system as previously reported [10]. The intensity of the DAF-2DA green fluorescence in ACh-treated cells was measured and compared with that in non-treated cells (λ_{Ex} 492 nm; λ_{Em} 515 nm).

2.7. Transfection

To investigate the direct contribution of Akt phosphorylation to HIF-1α stabilization or that of HIF-1α to the ACh effect, HEK293 cells were transfected with an expression vector for wild-type Akt (wt Akt), dominant-negative Akt (dn Akt) [11], wild-type HIF-1α (wt HIF-1α) [12] or dominant-negative HIF-1α (dn HIF-1α), using Effectene (Qiagen, Valencia, CA, USA) according to the manufacture's protocol. After transfection, HEK293 cells were pretreated with 1 mM ACh for 12 h, followed by evaluating the HIF-1α protein level or by hypoxia for 12 h and MTT activity in each group was evaluated. As a control, cells were transfected with a vector for green fluorescent protein (GFP).

2.8. RT-PCR

Total RNA was isolated from H9c2 cells according to a modified acid guanidinium–phenol–chloroform method using an RNA isolation kit (ISOGEN; Nippon Gene, Tokyo, Japan), and reverse-transcribed to obtain a first-strand cDNA. This first-strand cDNA was amplified by specific primers for HIF-1α, and the PCR products were fractionated by electrophoresis.

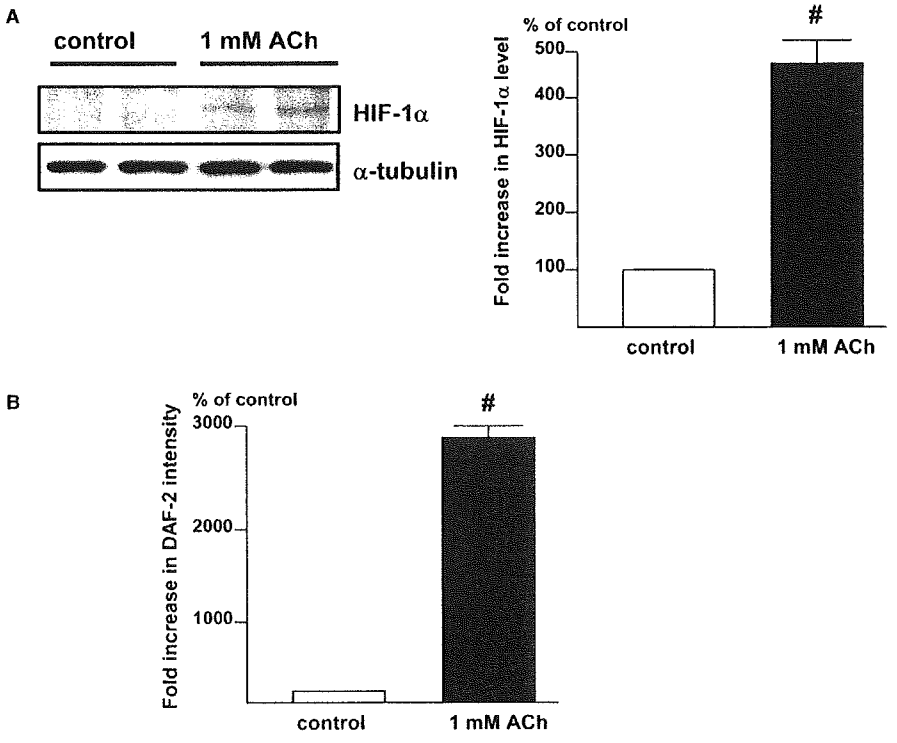

Fig. 1. HIF-1α is induced by ACh in rat cardiomyocytes even under normoxia. (A) After treatment of H9c2 cells with 1 mM ACh for 8 h, the HIF-1α protein level is increased ($^{\#}P < 0.05$ vs. control, $n = 4$). (B) ACh (1 mM) increases the intensity of DAF-2DA fluorescence ($^{\#}P < 0.01$ vs. control, $n = 3$).

2.9. Vagal nerve stimulation in myocardial ischemia

Left ventricular myocardial ischemia (MI) was performed by 3 h of left coronary artery (LCA) ligation in anesthetized 9-week-old male Wistar rats under artificial ventilation previously described [2]. Sham-operated (control) rats did not undergo LCA ligation. For vagal nerve stimulation (VS), the right vagal nerve in the neck was isolated and cut. Only the distal end of the vagal nerve was stimulated in order to exclude the effects of the vagal afferent. The electrode was connected to an isolated constant voltage stimulator. VS was performed from 1 min before the LCA ligation until 3 h afterwards, using 0.1 ms pulses at 10 Hz (MI-VS). The electrical voltage of the pulses was adjusted to obtain a 10% reduction in the heart rate before LCA ligation, but VS (MI-VS) was not associated with any blood pressure reduction during the experiments, compared with MI. At the end of the experiments, the rats were either injected with 2 ml of 2% Evans blue dye via the femoral vein to measure the risk area followed by determination of the infarct size with 2% triphenyl tetrazolium chloride (TTC) staining or the heart was excised for protein isolation and subsequent Western Blotting to detect HIF-1α protein. The percentage of the infarcted area of the left ventricle was calculated as the ratio of the infarcted area to the risk area.

2.10. Densitometry

The Western Blotting data were analyzed using Kodak 1D Image Analysis Software (Eastman Kodak Co., Rochester, NY, USA).

2.11. Statistics

The data were presented as means ± S.E. The mean values between two groups were compared by the unpaired Student's *t* test. Differences among data were assessed by ANOVA for multiple comparisons of results. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Posttranslational regulation of HIF-1α by ACh through a non-hypoxic pathway

ACh (1 mM) increased HIF-1α protein expression in H9c2 cells under normoxia (Fig. 1A). ACh increased NO production, as evaluated by DAF-2DA (Fig. 1B), suggesting that

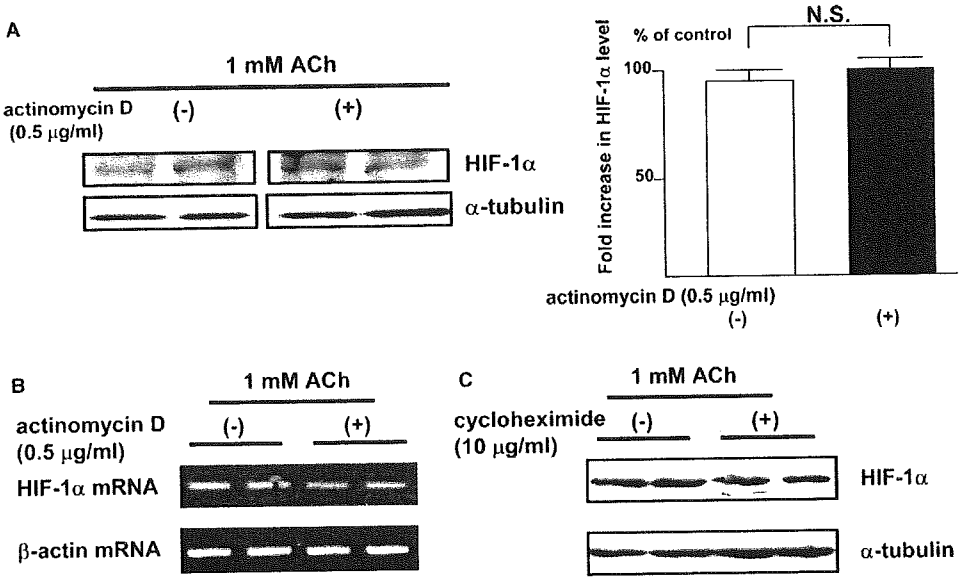

Fig. 2. HIF-1α induction by ACh is posttranslationally regulated in rat cardiomyocytes under normoxia. (A) The HIF-1α protein level in H9c2 cells in the presence of 0.5 μg/ml of actinomycin D is increased by 1 mM ACh to a comparable level to that in the absence of actinomycin D (N.S., not significant, $n = 3$). (B) Actinomycin D does not decrease the HIF-1α mRNA level, as evaluated by RT-PCR. (C) Cycloheximide (10 μg/ml) does not affect the HIF-1α protein level ($n = 3$).

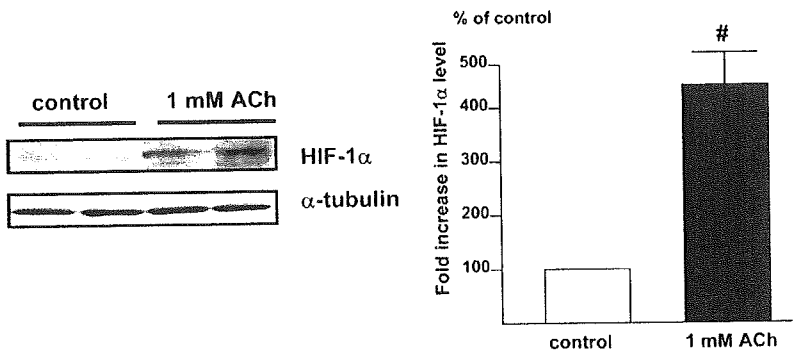

Fig. 3. Rat primary cultured cardiomyocytes show comparable HIF-1α induction by 1 mM ACh to that in H9c2 cells ($^{\#}P < 0.05$ vs. control, $n = 3$).

NO is involved in the signal transduction of HIF-1α induction. Actinomycin D (0.5 μg/ml; Figs. 2A and B) and cycloheximide (10 μg/ml; Fig. 2C) did not decrease the HIF-1α level under normoxia, suggesting that HIF-1α degradation is regulated by ACh. Furthermore, ACh increased HIF-1 α level in primary cardiomyocytes without reducing their beating rate (Fig. 3). Since H9c2 cells did not beat, these results suggest that HIF-1 induction is independent of the heart rate-decreasing effect of ACh.

3.2. Akt phosphorylation by ACh

ACh had no effect on the total Akt protein level, but increased Akt phosphorylation (Fig. 4A) as effectively as SNAP (data not shown). The ACh-induced Akt phosphorylation was inhibited by atropine in a dose-dependent manner (Fig. 4B). ACh-induced Akt phosphorylation and its inhibition by atropine were also observed in rat primary cardiomyocytes (Fig. 4C).

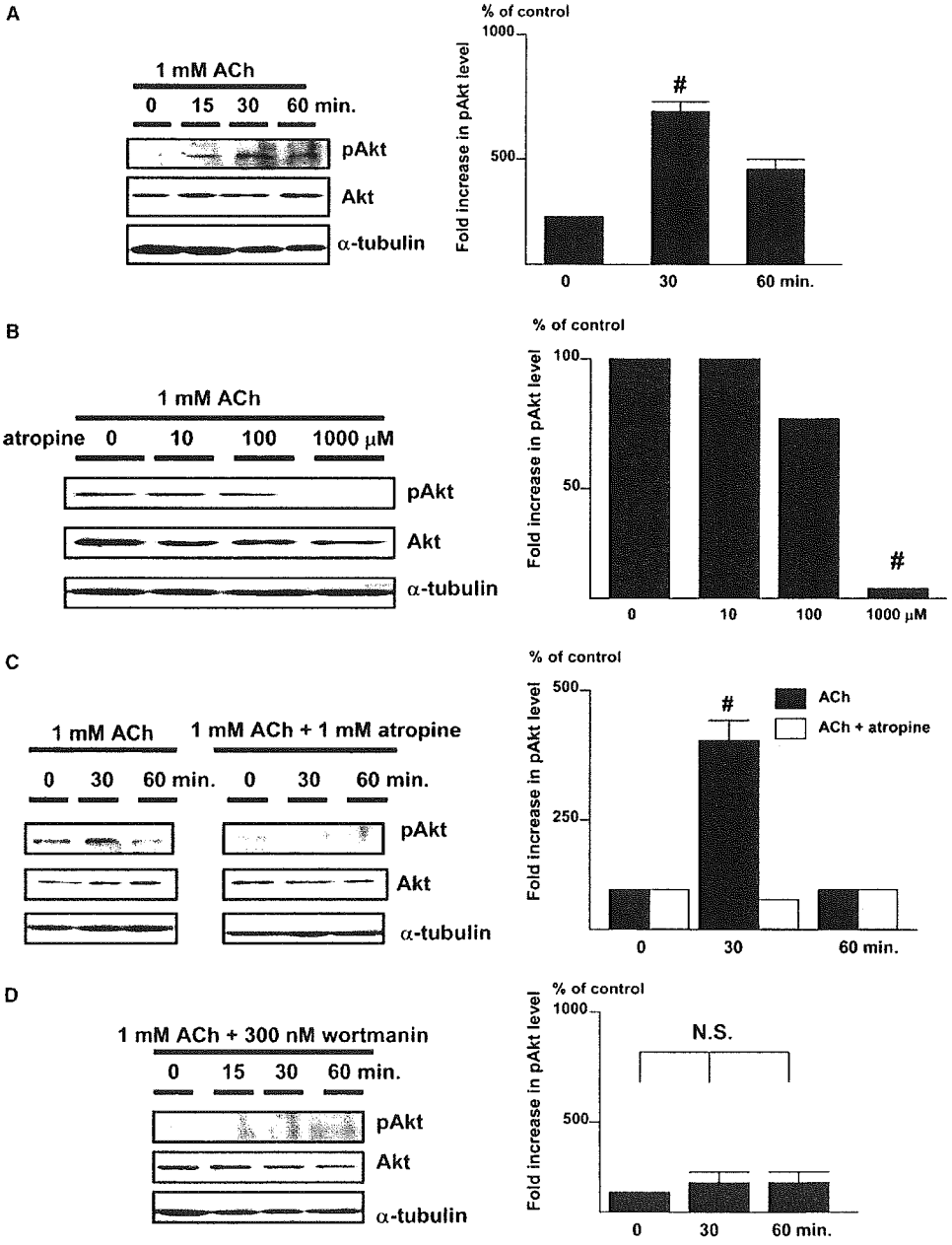

Fig. 4. Akt is activated by ACh in rat cardiomyocytes, leading to HIF-1α induction. (A) Akt phosphorylation in H9c2 cells is rapidly increased by 1 mM ACh ($^{\#}P < 0.05$ vs. baseline, $n = 4$), whereas the total protein level of Akt remains unaffected. (B) The ACh-induced increase in Akt phosphorylation is blocked by 1 mM atropine ($^{\#}P < 0.05$ vs. 0 μM atropine, $n = 3$). (C) ACh (1 mM) also increases Akt phosphorylation in rat primary cardiomyocytes ($^{\#}P < 0.05$ vs. baseline, $n = 3$), and atropine blocks this effect. (D) Pretreatment with 300 nM wortmannin completely inhibits ACh-induced Akt phosphorylation in H9c2 cells (N.S., not significant, $n = 3$). (E) Wortmannin (300 nM) also inhibits HIF-1α induction by ACh ($^{\#}P < 0.05$ vs. wortmannin (+), $n = 3$). Each figure shows a representative result from three independently performed experiments ($n = 3$). (F) In contrast to wt Akt, HIF-1α induction by ACh is blocked by dn Akt in HEK293 cells ($n = 4$).

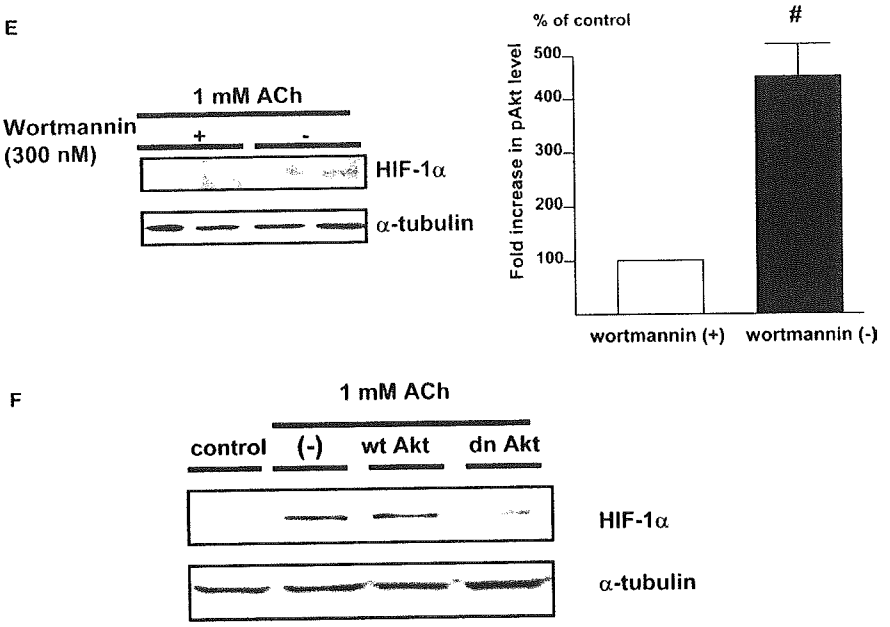

Fig. 4 (*continued*)

3.3. PI3K/Akt Pathway for HIF-1α induction by ACh

Wortmannin completely inhibited the ACh-induced Akt phosphorylation (Fig. 4D), in clear contrast to the data in Fig. 4A. Furthermore, it also attenuated the HIF-1α induction by ACh (Fig. 4E). To elucidate the contribution of Akt phosphorylation to HIF-1α protein level in normoxia, dn Akt was introduced into HEK293 cells, and found to partially inhibit the HIF-1α induction by ACh (Fig. 4F).

3.4. Effect of ACh on apoptosis during hypoxia

The DePsipher assay clearly showed that hypoxia (1% oxygen concentration) for 12 h caused mitochondrial membrane potential collapse leading to cell death, and that 1 mM ACh inhibited this collapse in H9c2 cells (Fig. 5A). ACh attenuated the decrease in MTT activity caused by 12 h of hypoxia in H9c2 cells (Fig. 5B; 103.4 ± 0.8% in ACh + hypoxia vs. 56.6 ± 0.7% in hypoxia, $P < 0.01$, $n = 8$) and HEK293 cells ($P < 0.01$ vs. hypoxia). The caspase-3 activity was increased by hypoxia in H9c2 cells, and pretreatment with 1 mM ACh inhibited this increase (Fig. 5C; 128 ± 2% in hypoxia vs. 90 ± 2% in ACh + hypoxia, $P < 0.01$, $n = 4$). To elucidate the dependency of the ACh-induced protective effect on HIF-1α, dn HIF-1α was transfected into HEK293 cells, followed by ACh pretreatment and then hypoxia. It was found that dn HIF-1α inhibited the protective effect of ACh from hypoxia (Fig. 5D; 115.1 ± 1.2% in wt HIF-1α and 111.8 ± 1.8% in GFP vs. 59.0 ± 3.4% in dn HIF-1α, $P < 0.05$, $n = 10$), suggesting that HIF-1α induction by ACh is partially responsible for the protective effect.

3.5. Effect of vagal stimulation on HIF-1α in myocardial ischemia

To evaluate the significance of ACh for cardioprotection in vivo, the vagal nerve was stimulated prior to the MI. Histological analysis demonstrated a tendency for the infarcted area from the vagal nerve-stimulated (MI-VS) hearts to be smaller than that from non-stimulated (MI) hearts (31.5 ± 4.6% in MI-VS vs. 40.9 ± 2.5% in MI, $n = 3$), even though the risk areas (non-perfused areas) were comparable (Fig. 6A; 59.2 ± 1.0% in MI-VS vs. 53.7 ± 1.0% in MI, $n = 3$). In the MI-VS hearts, the HIF-1α protein level was further elevated compared to that in the MI hearts (Fig. 6B; 244 ± 24% in MI-VS vs. 112 ± 1% in MI, $n = 3$). These results suggest that vagal nerve stimulation in the ischemic heart activates both the hypoxic and non-hypoxic pathways of HIF-1α induction, resulting in increased induction of HIF-1α.

3.6. Non-hypoxic induction of HIF-1α in other cells

The observed ACh-mediated HIF-1 induction was not limited to H9c2 or primary cultured cardiomyocytes, but also found in several other types of cell lines, including HEK293, and HeLa cells (Fig. 7). Since these cells did not beat spontaneously, the results suggest that the system of ACh-mediated HIF-1α induction is not only independent of the beating rate of cardiomyocytes, but also a generally conserved system in cells.

4. Discussion

4.1. Cardioprotective action by ACh and vagal stimulation via the muscarinic receptor

Using animal models, several studies have shown that accentuated antagonism against the sympathetic nervous system is a major mechanism for the beneficial effect of vagal tone on the ischemic heart [13]. Although ACh was involved in triggering preconditioning mechanisms in an ischemia-reperfusion model [3], it remained unclear whether vagal nerve stimulation in acute ischemia or hypoxia followed these mechanisms. In the present study, we have disclosed that ACh possesses a

protective effect on cardiomyocytes. In rat cardiomyocytes, ACh triggered a sequence of survival signals through Akt that eventually induced HIF-1α, inhibited the collapse of the mitochondrial membrane potential and decreased caspase-3 activity, thereby leading to the survival of cardiomyocytes under hypoxia. Furthermore, our results suggest ACh exerts this action through Akt in other cells. The current study therefore provides another insight into the cellular mechanism for the cardioprotective effects of ACh and vagal stimulation.

4.2. Signaling pathway of ACh via PI3K/Akt and antiapoptotic effects of ACh

Since previous studies demonstrated that a PI3K inhibitor greatly reduced HIF-1α induction in heart and renal cells [14,15] and a few studies have reported that MAP kinase is activated through ACh, we focused on the PI3K/Akt pathway, one of the important cell survival signaling pathways [16], and found that ACh directly activated Akt phosphorylation via PI3K. PI3K/Akt signaling has been reported to have an

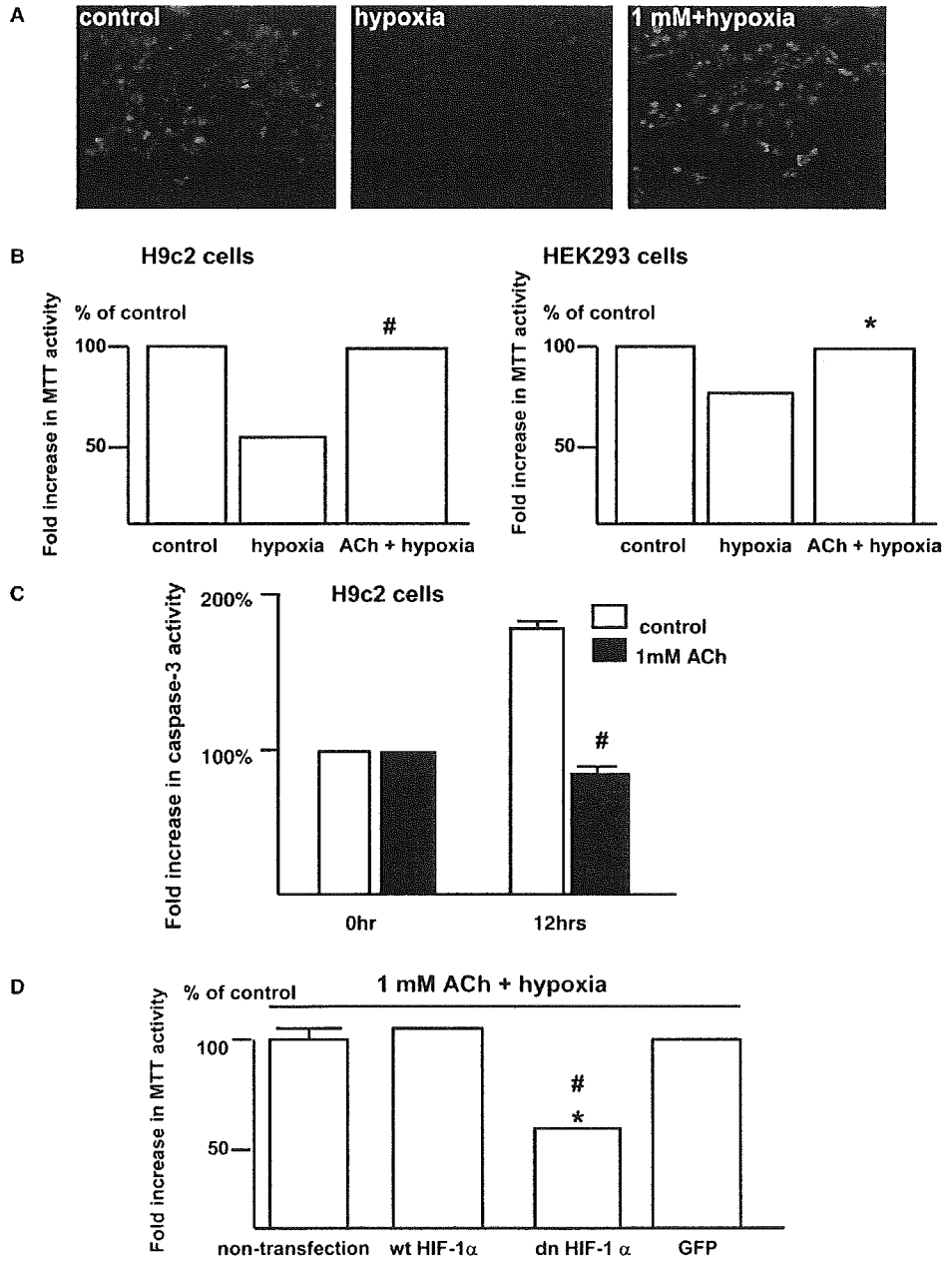

Fig. 5. Collapse of the mitochondrial membrane potential in rat cardiomyocytes under hypoxia is attenuated by ACh pretreatment. (A) Hypoxia decreases the mitochondrial membrane potential in H9c2 cells within 12 h. Red spots are decreased by hypoxia, whereas pretreatment with 1 mM ACh for 12 h inhibits this effect. (B) Pretreatment with 1 mM ACh inhibits the decrease in MTT reduction activity induced by 12 h of hypoxia not only in H9c2 cells ($^{\#}P < 0.01$ vs. hypoxia, $n = 8$) but also in HEK293 cells ($^{*}P < 0.01$ vs. hypoxia, $n = 8$). (C) Hypoxia increases caspase-3 activity, whereas pretreatment with 1 mM ACh inhibits this effect ($^{\#}P < 0.01$ vs. hypoxia, $n = 3$). (D) In contrast to wt HIF-1α or GFP, dn HIF-1α alone decreases the MTT activity under hypoxia after ACh treatment ($^{\#}P < 0.01$ vs. wt and GFP, $^{*}P < 0.05$ vs. non-transfection, $n = 10$).

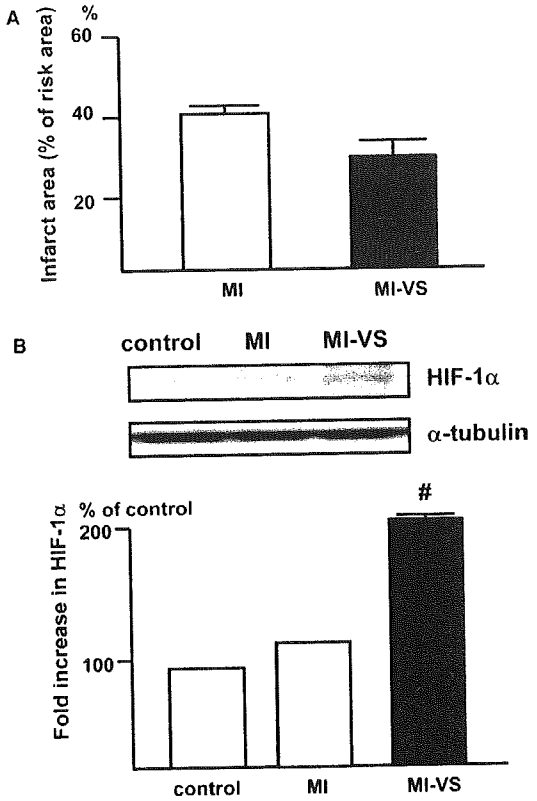

Fig. 6. Vagal nerve stimulation decreases infarcted area with increased HIF-1α expression. (A) A quantitative analysis reveals comparable non-perfused areas in both vagal-stimulated (MI-VS) and non-stimulated (MI) hearts, whereas the infarcted area identified by TTC staining is smaller in the MI-VS heart than in the MI heart. (B) HIF-1α induction in the ischemic heart is increased by vagal stimulation (MI-VS) compared with that in ischemia alone (MI) ($^{\#}P < 0.01$ vs. MI) ($n = 3$).

antiapoptotic activity through various features, such as inhibition of Bad-binding to Bcl-2, caspase 9, Fas and glycogen synthetase kinase-3 [17,18]. These facts imply a definite involvement of Akt activation in cell survival. As shown using dn HIF-1α, ACh inhibited hypoxia-induced cell death through HIF-1α induction via Akt phosphorylation. These results indicate that ACh actually protects cardiomyocytes from hypoxia at the cellular level.

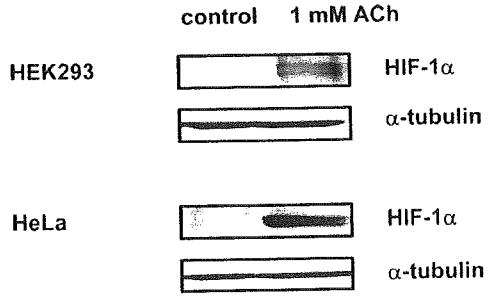

Fig. 7. HIF-1α is induced by ACh under normoxia in other cells. ACh (1 mM) increases HIF-1α protein level in HEK293 and HeLa cells ($n = 3$ each) under normoxia.

4.3. Additional induction of HIF-1α by ACh and vagal stimulation

HIF-1α regulates the transcriptional activities of very diverse genes involved in cell survival and is itself regulated at the posttranslational level by VHL [4,6,7]. Recent studies have shown that HIF-1α is also regulated through a non-hypoxic pathway involving angiotensin II, TNF-α and NO [8,9,19,20]. Therefore, it is speculated that cardiomyocytes possess a similar system for regulating HIF-1α through ACh, independent of the oxygen concentration. Induction of HIF-1α is a powerful cellular response against hypoxia, and further increases in its expression by other pathways may be beneficial. The present results indicate that the significance of ACh or vagal nerve stimulation in hypoxic stress can be attributed to additional HIF-1α induction through dual induction pathways, i.e., hypoxic and non-hypoxic pathways.

The present study has revealed that ACh-mediated HIF-1α induction is widely conserved in other cells. Consistent with a previous report [10], the current results suggest that NO is produced by ACh. According to a report that NO attenuates the interaction between pVHL and HIF-1α through inhibiting PHD activity [21], it is possible that ACh may increase the HIF-1α protein level through NO. Recent studies conducted by Krieg et al. [3] and Xi et al. [22], have provided supportive data compatible with our results, while another study by Hirota et al. [23] also revealed a non-hypoxic pathway for HIF-1α induction by ACh in a human kidney-derived cell line.

The signaling pathway of the muscarinic receptor has been studied extensively, and many pathways are involved in its specific biological effects. Therefore, possible involvement of other pathways in the non-hypoxic induction of HIF-1α cannot be excluded. However, it was demonstrated that dn Akt and dn HIF-1α decreased the effect of ACh. Consistent with a recent study [24], we have revealed that ACh or vagal stimulation protects cardiomyocytes in the acute phase. This observation suggests that the protective effect in the acute phase may result in inhibition of cardiac remodeling in the chronic phase, since vagal stimulation produces additional HIF-1α induction through a non-hypoxic pathway, which increases cell survival.

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Hypoxia-Inducible Factor-1α Is Involved in the Attenuation of Experimentally Induced Rat Glomerulonephritis

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Key Words
Glomerulonephritis · Habu snake venom · Angiotensin II · Hypoxia-inducible factor-1α

Abstract
Background/Aim: Among various kidney disease models, there are few rat glomerulonephritis (GN) models that develop in a short time, and with mainly glomerular lesions. Hypoxia-inducible factor (HIF)-1α is a transcriptional factor that induces genes supporting cell survival, but the involvement of HIF-1α in attenuating the progression of GN remains to be elucidated. We developed a new model of rat GN by coadministration of angiotensin II (AII) with Habu snake venom (HV) and investigated whether HIF-1α is involved in renal protection. ***Methods:*** Male Wistar rats were unilaterally nephrectomized on day 1, and divided into 4 groups on day 0; N group (no treatment), HV group, A group (AII), and H+A group (HV and AII). To preinduce HIF-1α, cobalt chloride ($CoCl_2$) was injected twice before injections of HV and AII in 11 rats. ***Results:*** GN was detected only in the H+A group; observed first on day 2 and aggravated thereafter. HIF-1α was expressed in the glomeruli and renal tubules in the A and H+A groups. In the H+A group, GN was remarkably reduced by $CoCl_2$ pretreatment (44.9 to 12.2%, $p < 0.01$). ***Conclusion:*** Both HV and AII were critical for the development of GN, and HIF-1α remarkably attenuated the progression of GN.

Introduction

Many animal studies have been performed in attempts to overcome the poor prognosis of chronic renal failure due to diabetic nephropathy and glomerulonephritis (GN) [1–5]. Although factors involved in the pathogenesis of GN have been intensively investigated, the development of a proper animal GN model with high reproducibility and simplicity as well as a model without time-consuming process is required. Experimental rat models of GN are classified into several groups in terms of the pathophysiological mechanisms of renal diseases. Anti-glomerular basement membrane nephritis was developed with depositions of immune complex using anti-glomerular basement membrane antibody [3, 6], tubulointerstitial injury was caused by cyclosporine A [4] and injury of renal tubules by ischemia [5]. However, there are few rat GN models with mainly pathological features in the glo-

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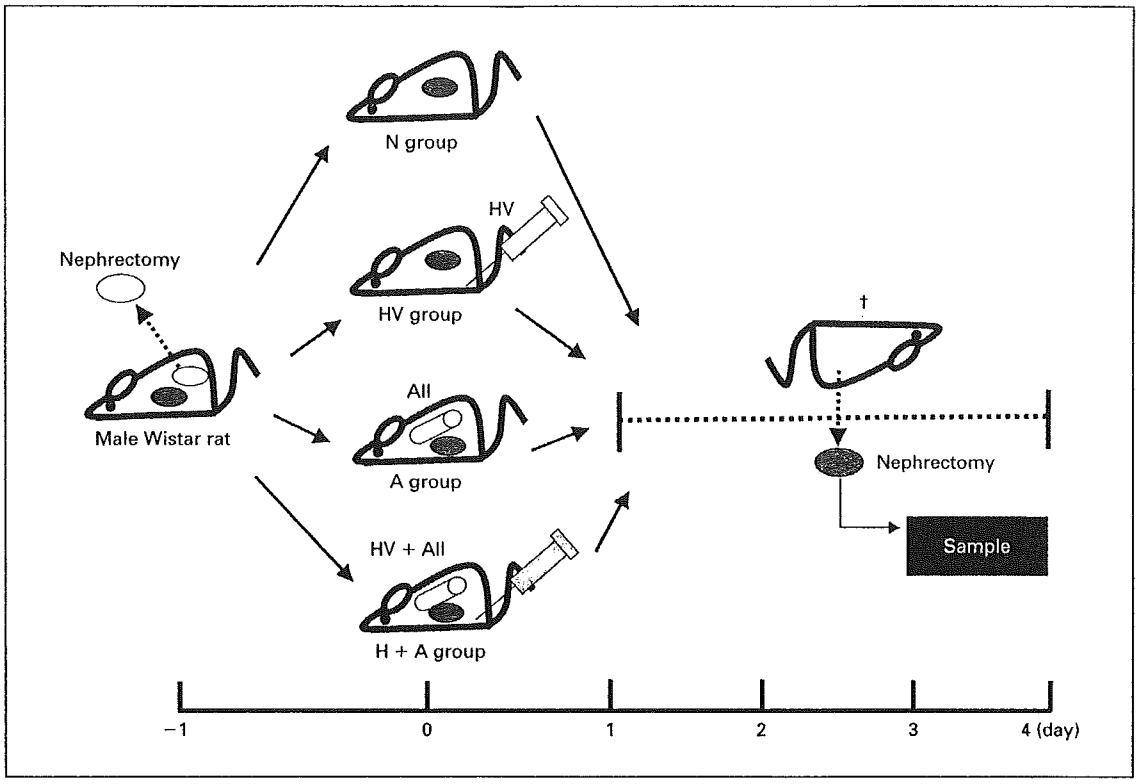

Fig. 1. Study Protocol. All rats are unilaterally nephrectomized on day 1 and divided into 4 groups on day 0. N group: no injection of reagents. HV group: injection of 3.5 mg/kg of Habu snake venom (HV). A group: continuous administration of 100 ng/min of angiotensin II (AII). H+A group: administration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

meruli that are developed in a short time [7]. Angiotensin II (AII) is known to increase blood pressure through vascular contraction, and to be profoundly involved in cardiovascular hypertrophy and the contraction of intrarenal arteries. AII is also directly involved in the progression of glomerulosclerosis via the effect of hyperfiltration with or without hypertension [8, 9]. Many studies have revealed important factors involved in the pathogenesis of GN or factors aggravating GN, but evaluating further factors that suppress the occurrence of GN is also crucial. To investigate the features of renal protection, we focused on hypoxia-inducible factor (HIF)-1α. HIF-1α, a transcriptional factor with formation of a heterodimer with HIF-1β [10], is post-transcriptionally regulated and its protein level is elevated by hypoxia through inhibition of ubiquitin-mediated degradation. HIF-1α is known to be a survival factor responsible for inducing lines of genes supporting cell survival such as glucose metabolism (glucose transporters and glycolytic enzymes), vasomotor regulation (heme oxygenase-1 and endothelin-1), angiogenic growth (vascular endothelial growth factor), and anemia control (erythropoietin and transferrin) [11–13]. Recent studies have demonstrated that non-hypoxic stimuli like AII can also activate HIF-1α [14, 15], but the role of HIF-1α induction in attenuating the progression of GN remains to be elucidated. Accordingly, we developed a new rat GN model by coadministration of AII with Habu snake venom (HV) and investigated whether preinduction of HIF-1α leads to renal protection.

Materials and Methods

Development of Rat GN Model

All experiments were approved by the institutional review board for the care of animal subjects and were performed in accordance with guidelines of Kochi Medical School. Nine-week-old male Wistar rats (180–220 g) were purchased from Japan SLC (Shizuoka, Japan). Rats were unilaterally nephrectomized on day 1. On day 0, the rats were divided into 4 groups. In the first group, no treatment was performed with any reagents or surgical procedure (N group, n = 6). In the second group, rats were injected with 3.5 mg/kg of HV (Sigma-Aldrich Co., Steinheim, Germany) through the femoral vein (HV group, n = 11). In the third group, rats were continuously adminis-