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Full Paper

Acetylcholine Inhibits the Hypoxia-Induced Reduction of Connexin43 Protein in Rat Cardiomyocytes

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Abstract. In a recent study, we demonstrated that vagal stimulation increases the survival of rats with myocardial infarction by inhibiting lethal arrhythmia through regulation of connexin43 (Cx43). However, the precise mechanisms for this effect remain to be elucidated. To investigate these mechanisms and the signal transduction for gap junction regulation, we investigated the effect of acetylcholine (ACh), a parasympathetic nerve system neurotransmitter, on the gap junction component Cx43 using H9c2 cells. When cells were subjected to hypoxia, the total Cx43 protein level was decreased. In contrast, pretreatment with ACh inhibited this effect. To investigate the signal transduction, cells were pretreated with L-NAME, a nitric oxide synthase inhibitor, followed by ACh and hypoxia. L-NAME was found to suppress the ACh effect. However, a NO donor, SNAP, partially inhibited the hypoxia-induced reduction in Cx43. To delineate the mechanisms of the decrease in Cx43 under hypoxia, cells were pretreated with MG132, a proteasome inhibitor. Proteasome inhibition produced a striking recovery of the decrease in the total Cx43 protein level under hypoxia. However, cotreatment with MG132 and ACh did not produce any further increase in the total Cx43 protein level. Functional studies using ACh or okadaic acid, a phosphatase inhibitor, revealed that both reagents inhibited the decrease in the dye transfer induced by hypoxia. These results suggest that ACh is responsible for restoring the decrease in the Cx43 protein level, resulting in functional activation of gap junctions.

Keywords: acetylcholine, connexin43, cardiomyocyte, hypoxia, proteasome inhibitor

Introduction

The prognosis of patients with chronic heart failure remains poor, despite the introduction of new pharmacological approaches and defibrillation devices, mainly due to lethal arrhythmia (1). Therefore, another therapeutic approach would be indispensable. In heart failure, the sympathetic nerve system is relatively activated compared with the parasympathetic nerve system (2), and this sympathetic nerve system-predominant condition is known to be involved in arrhythmogenicity. Recently, vagal nerve stimulation was reported to remarkably improve the survival rate of rats with heart failure due to myocardial infarction (3), suggesting that reactivation of the parasympathetic nerve system, which is suppressed in heart failure, plays a crucial role in attenuating the progression of heart failure. Moreover, our recent study revealed that acetylcholine (ACh), a parasympathetic nerve system neurotransmitter, plays an important role in regulating the protein level of the gap junction component connexin43 (Cx43) in the infarcted heart and cardiomyocytes under hypoxia (4). However, the precise mechanisms by which ACh regulates Cx43 remain to be elucidated. To investigate these mechanisms, we focused on Cx43 in H9c2 cells.

Gap junctions are intercellular junctions, and several connexin family members, including Cx43, participate in their formation. Among the connexin family members, Cx43 is the principal electrical coupling

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protein in ventricles, while Cx40 plays the same role in atria. The functions of Cx43 are regulated by phosphorylation as well as the protein level. Cx43 phosphorylation can modulate the channel properties and turnover dynamics. SDS-PAGE of Cx43 generally reveals a faster non-phosphorylated isoform (NP-Cx43) and slower phosphorylated isoforms (P-Cx43). Cx43 is synthesized in the rough endoplasmic reticulum, transported to the Golgi apparatus, and ultimately trafficked to the plasma membrane (5, 6). Recent evidence has suggested that Cx43 is involved in modifying arrhythmogenic conditions (7, 8) since Cx43 knockout mice were subject to sudden death caused by lethal arrhythmia, including ventricular tachycardia, or fibrillation (9, 10). Although many other factors, including sodium, potassium, and calcium channels, appear to be involved in arrhythmogenicity, it is speculated that functional deletion of Cx43 is also responsible for arrhythmia. To date, it has remained unclear whether and how ACh modulates Cx43. Therefore, we focused on the effect of ACh on Cx43.

Materials and Methods

Cell culture and pharmacological agents

H9c2 cells, which are spontaneously immortalized ventricular myoblasts from rat embryos, were used due to their conserved electrical and signal transduction characteristics (11). The cells were cultured in DMEM supplemented with 10% FBS and antibiotics. H9c2 cells were pretreated with 1 mM ACh for 8 h, followed by 1 h of hypoxia (1% of oxygen concentration). We chose N^{ω}-nitro-L-arginine methyl ester (L-NAME) (Sigma Chemical Co., St Louis, MO, USA), a specific nitric oxide (NO) synthase inhibitor, to determine whether NO mediates the signal transduction for Cx43 expression. L-NAME (1 mM) was administered for 1 h together with ACh, followed by hypoxia for 1 h. H9c2 cells were also treated with 1 mM *S*-nitroso-*N*-acetyl-l,l-penicillamine (SNAP) (Sigma Chemical Co.) before hypoxia. We used 10 μM Cbz-leu-leu-leucinal (MG132) (Sigma Chemical Co.) or 1 μM okadaic acid to investigate whether hypoxia enhanced Cx43 degradation or phosphorylation was important for regulating the functional activity of Cx43.

Western blot analysis

Cells were harvested from the dishes and prepared for immunoblotting as described previously (8). After washing in PBS, cells were lysed with SDS sample buffer and boiled for 10 min. After electrophoresis in a 10% SDS-polyacrylamide gel, proteins were transferred to a polyvinylidene difluoride membrane. The membrane was soaked in 4% skim milk in TBST solution overnight, then incubated with an anti-Cx43 polyclonal antibody (ZYMED Laboratories, Inc., South San Francisco, CA, USA) for 1 h, thoroughly washed, and then incubated with an anti-rabbit IgG secondary antibody (BD Transduction Laboratories, San Diego, CA, USA) for 40 min. Finally, the membrane was washed and subjected to chemiluminescent detection using the ECL Plus Western Blotting Detection Reagents (Amersham Biosciences, Piscataway, NJ, USA). We performed repeatedly 3-5 times each experiment using duplicate samples. The Western blotting data were analyzed using Kodak 1D Image Analysis Software (Eastman Kodak Co., Rochester, NY, USA).

Immunohistochemistry

H9c2 cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with 1% Triton X-100 for another 10 min. To block nonspecific antibody binding, cells were incubated with 5% skim milk and successively incubated with an anti-Cx43 polyclonal antibody (ZYMED Laboratories, Inc.), in 1% skim milk at 4°C overnight and then with a Cy3-labeled secondary antibody (Jackson ImmunoReserch Laboratories, West Grove, PA, USA) at 4°C overnight. Actin staining was performed using FITC-conjugated phalloidin and then examined with a laser scanning confocal microscope.

Functional analysis of gap junction using a scrape and scratch method

A scrape-loading method can be used to introduce macromolecules into cultured cells by inducing a transient tear in the plasma membrane without affecting cell viability, thereby allowing sensitive determination of cell-cell communication. Following the treatment with ACh or okadaic acid, cells cultured on a coverslip were rinsed with PBS, and then 1% Lucifer Yellow was applied to the center of the converslip. A 27 gauge needle was used to create two longitudinal scratches through the cell monolayer. The cells were incubated in the dye mix for exactly 1 min, quickly rinsed three times with PBS, and finally examined by fluorescence microscopy. Lucifer Yellow does not diffuse through intact plasma membranes, but its low molecular weight permits its transmission from one cell to another, presumably across patent gap junctions (12 – 16). The area of the dye transferred from the scratched margin in hypoxia or hypoxia with ACh treatment was semi-quantified using the NIH image system and compared with that in normoxia.

Statistical analyses

Data are presented as the mean ± S.E.M. Differences

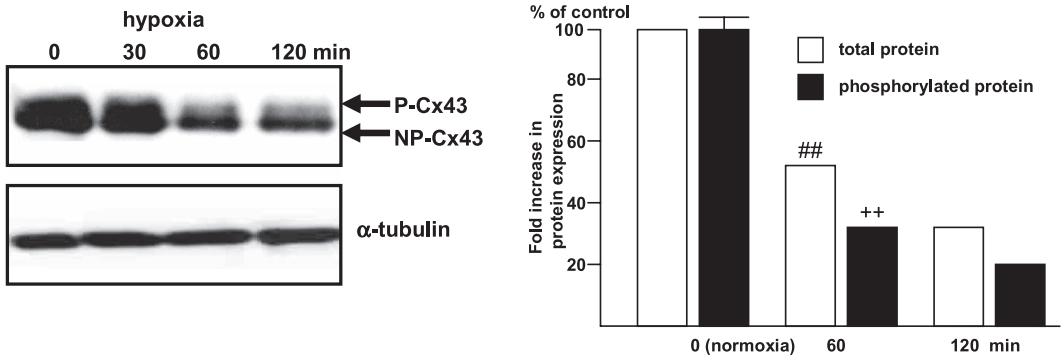

Fig. 1. Cx43 phosphorylation is decreased by hypoxia. Cells are subjected to 30 – 120 min of hypoxia and then analyzed by Western blot analysis. Cx43 phosphorylation (P-Cx43) is reduced to 32 ± 4% of the level under normoxia ($^{++}P<0.01$ *vs* 0 min, $^{\#\#}P<0.01$ *vs* 0 min) by 1% hypoxia, and the effect is remarkable after 60 min of hypoxia. NP-Cx43: non-phosphorylated form of Cx43. Open bars: total Cx43 protein level, closed bars: P-Cx43 level. Representative data from 5 independently performed experiments are shown (n = 5).

were assessed by ANOVA followed by Fisher's PLSD for multiple comparisons. The results were considered statistically significant at the level of $P<0.05$.

Results

Hypoxia decreases the Cx43 protein level in H9c2 cells

Several different forms of Cx43 were observed in the case of H9c2 cell (Fig. 1). The upper bands represented the phosphorylated forms, while the lower band corresponded to the non-phosphorylated form. We examined the acute effect of hypoxia on the total Cx43 protein level in H9c2 cells (n = 5). The total protein level of Cx43, including NP-Cx43 and P-Cx43, gradually decreased during hypoxia (Fig. 1), and 60 min of hypoxia induced a remarkable decrease in the total Cx43 protein level ($^{\#\#}P<0.01$ *vs* 0 min of hypoxia) and reduced its phosphorylation to 32 ± 4% of the normoxic level ($^{++}P<0.01$ *vs* 0 min of hypoxia). These results suggest that the total Cx43 protein level is rapidly decreased under hypoxia.

ACh increases the Cx43 protein level in H9c2 cells under normoxia or hypoxia

To determine whether ACh could modulate the Cx43 protein level after acute treatment, we initially treated H9c2 cells with 1 mM ACh under normoxia (n = 3). When the cells were stimulated with 1 mM ACh under normoxia, the Cx43 protein level was transiently increased ($^{++}P<0.01$ *vs* 0 min), followed by a rapid decrease, and then another peak was observed at 8 h (Fig. 2A). Next, to examine the effect of ACh on the hypoxia-induced decrease in Cx43, we pretreated H9c2 cells with 1 mM of ACh for 7 h, followed by 1 h of hypoxia (n = 6). Compared to the Cx43 level under hypoxia alone (hypoxia), the Cx43 protein level in ACh-pretreated H9c2 cells was not decreased under hypoxia (ACh + hypoxia), but instead was rather sustained ($^{\#\#}P<0.01$ *vs* hypoxia; ns, not significant *vs* normoxia; n = 6) (Fig. 2B). This ACh-mediated inhibition of the decrease in Cx43 under hypoxia was also observed by immunocytochemistry since hypoxia decreased the Cx43 immunoreactivity, and ACh inhibited the reduction (Fig. 2C).

Inhibition of the decrease in the Cx43 protein level during hypoxia by ACh occurs via NO

To further characterize the signal transduction for ACh-mediated inhibition of the reduction in the Cx43 protein level under hypoxia, we investigated the effects of chemicals on the Cx43 protein level (n = 5) (Fig. 3). Pretreatment with L-NAME (1 mM) for 1 h inhibited the ACh-induced recovery of the Cx43 protein level during hypoxia, suggesting that NO participates in regulating the Cx43 protein level ($^{\#}P<0.05$ *vs* ACh, n = 5). To further investigate whether the protein level was affected by NO, the cells were treated with 1 mM SNAP, a NO donor, instead of ACh. SNAP partially inhibited the reduction in the Cx43 protein level compared with L-NAME treatment, further suggesting that NO plays a partial role in modulating the protein level ($^{+}P<0.05$ *vs* L-NAME, n = 5).

Cx43 is degraded under hypoxia

To further investigate the mechanisms of the decrease in Cx43 under hypoxia, H9c2 cells were pretreated with the proteasome inhibitor MG132 (n = 5) for 10 and 60 min during hypoxia (Fig. 4A). The proteasome inhibition produced a striking recovery of the decreased total Cx43 protein level. MG132 inhibited the reduction

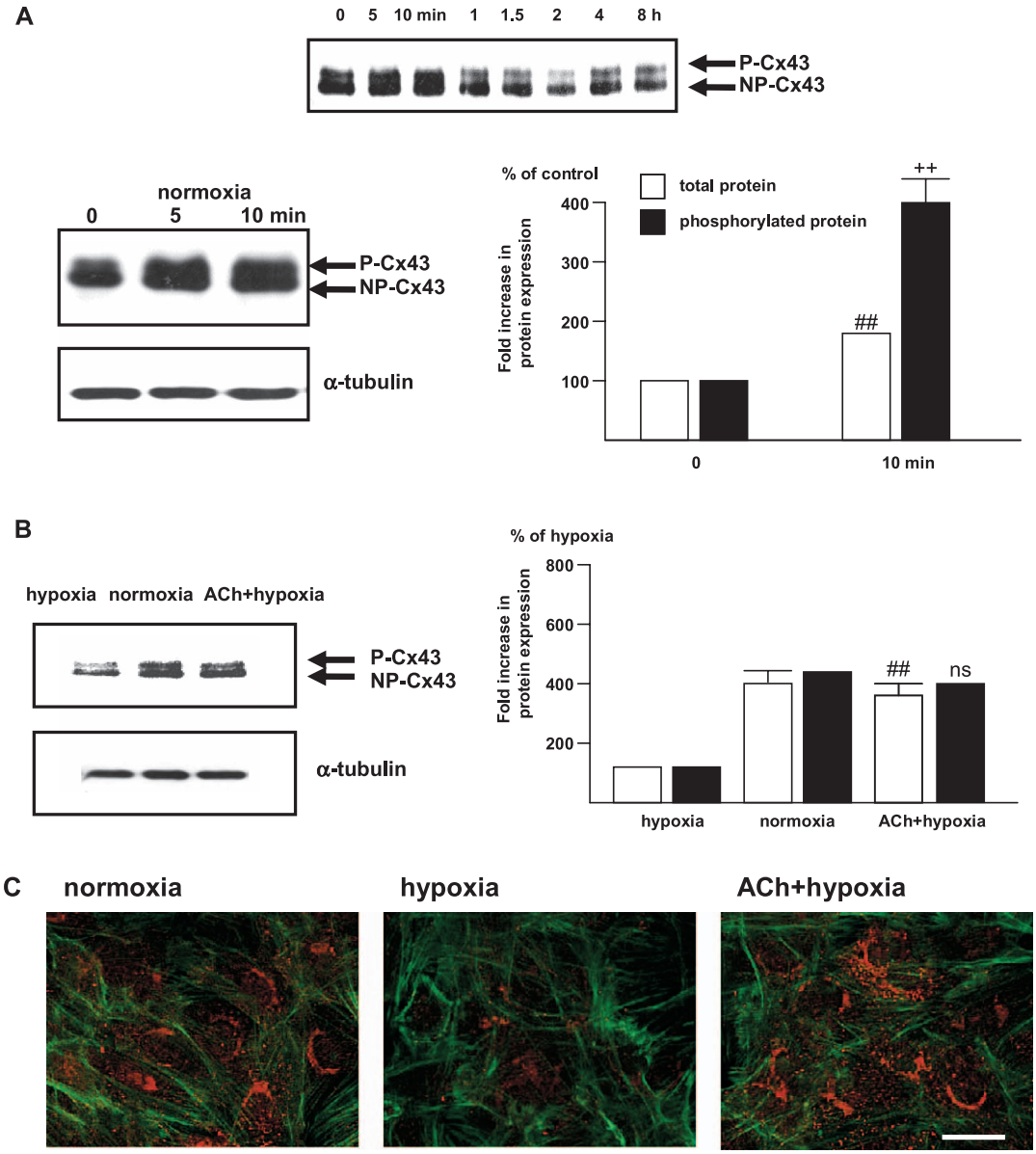

Fig. 2. ACh regulates Cx43 phosphorylation under normoxia and hypoxia. A: 1 mM ACh increases Cx43 phosphorylation (P-Cx43) in the acute phase under normoxia, reaching a peak of 409 ± 28% at 10 min ($^{++}P<0.01$ *vs* 0 min, $^{\#\#}P<0.01$ *vs* 0 min, n = 3). The entire time course shows another peak of the Cx43 protein level following the acute phase at 8 h. B: ACh suppresses the reduction in the Cx43 protein level induced by 1 h of hypoxia. ACh (1 mM) pretreated H9c2 cells show a sustained level of Cx43 phosphorylation, comparable to that under normoxia (normoxia), even under hypoxia (ACh + hypoxia) ($^{\#\#}P<0.01$ *vs* hypoxia; ns, not significant *vs* normoxia; n = 6). C: ACh inhibits the reduction in Cx43 immunoreactivity under hypoxia (red dots). Representative staining is shown. Cx43 is indicated by red dots. Bar: 50 μm.

in the Cx43 protein level by hypoxia for up to 60 min, suggesting that the reduction is due to activation of Cx43 protein degradation ($^{\#}P<0.05$ *vs* normoxia, n = 5). Furthermore, the effect of MG132 on inhibiting Cx43 degradation was not modified by ACh addition, and as a consequence, the effect of MG132 on inhibiting the hypoxia-induced decrease in Cx43 was comparable to that of cotreatment with MG132 and ACh (not significant *vs* ACh + MG132, n = 5) (Fig. 4B). These results suggest that ACh modulates the degradation process of Cx43.

ACh activates the function of gap junctions through an increase in the Cx43 protein level

To investigate whether ACh inhibition of the Cx43 protein level during hypoxia leads to functional recovery of gap junctions, we applied the scrape/scratch technique (n = 5). In a control experiment, scrape-loaded

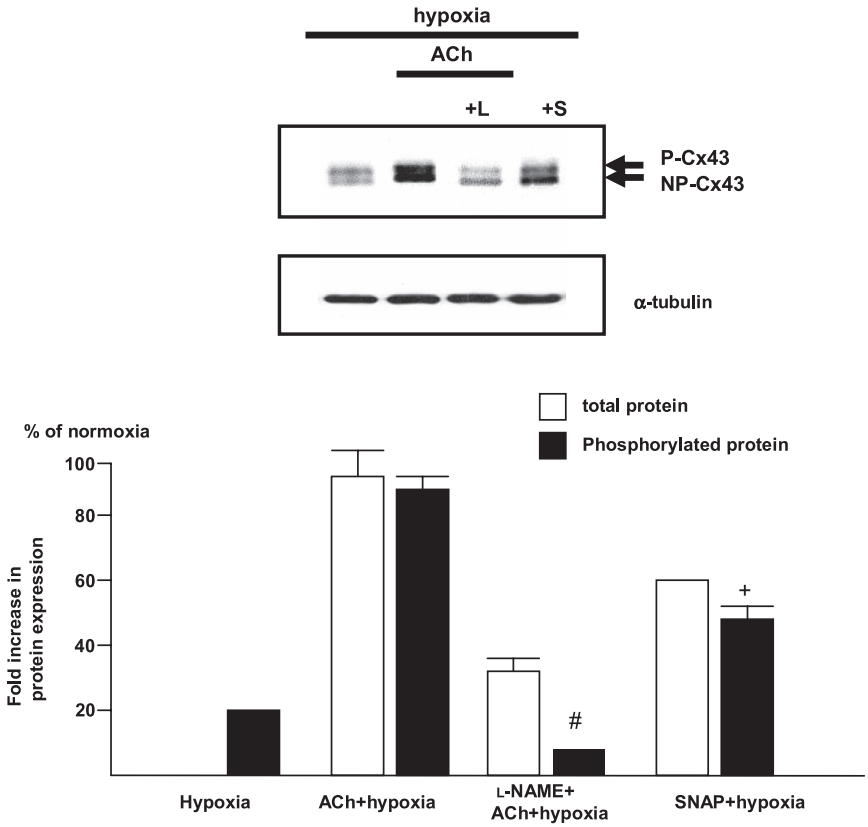

Fig. 3. NO is involved in the ACh signaling pathway that leads to the increase in Cx43 phosphorylation ($^{\#}P<0.05$ *vs* ACh + hypoxia; $^{+}P<0.05$ *vs* L-NAME + ACh + hypoxia). ACh: 1 mM ACh, L: 1 mM L-NAME, S: 1 mM SNAP. Representative data from 5 independently performed experiments are shown (n = 5).

cells in the presence of Lucifer Yellow showed positive transfer of Lucifer Yellow between cells. In contrast, cells treated with hypoxia appeared to lose their ability to communicate with each other and the dye transfer was blocked to $6 \pm 2\%$ of the intensity under normoxia. In contrast, ACh suppressed the hypoxia-induced blockage of dye transfer ($^{\#\#}P<0.01$ *vs* hypoxia, not significant *vs* normoxia, n = 5). The area of Lucifer Yellow fluorescence was increased in ACh-treated cells along the scraped margin during hypoxia ($62 \pm 10\%$ of the area under normoxia) (Fig. 5). These results suggest that hypoxia affects intercellular communication and that ACh functionally activates cell-cell communication, even under hypoxia, through increases in the Cx43 protein level. Furthermore, pretreatment with 1 μM okadaic acid, a phosphatase inhibitor, for 10 min recovered the reduction in the Cx43 protein level and the extent of dye transfer during hypoxia (Fig. 6). Taken together with the results obtained with the proteasome and phosphatase inhibitors, it is suggested that both the protein and phosphorylation levels of Cx43 are involved in the function of Cx43.

Discussion

In the current study, we have shown that the Cx43 protein level is regulated by ACh in the presence or absence of hypoxia. Even in normoxia, ACh regulated the Cx43 protein level and inhibited the reduction in the Cx43 protein level induced under hypoxia. Such modification of the Cx43 protein level by ACh partially occurred via NO, since the protein level sustained by ACh during hypoxia was affected by L-NAME, whereas SNAP showed similar effects to ACh. Furthermore, the results indicated that the hypoxia-induced decrease in the total Cx43 protein level is due to proteasome degradation. Taken together, these results further suggest that ACh is involved in inhibiting Cx43 degradation under hypoxia.

Our previous study revealed that vagal stimulation inhibited the reduction in the Cx43 protein level during acute myocardial ischemia and instead sustained a similar level to that in the normal heart (4). As a result, vagal stimulation was further shown to decrease the frequency of ventricular arrhythmia. Moreover, ACh sustained the dye transfer level, which was attenuated

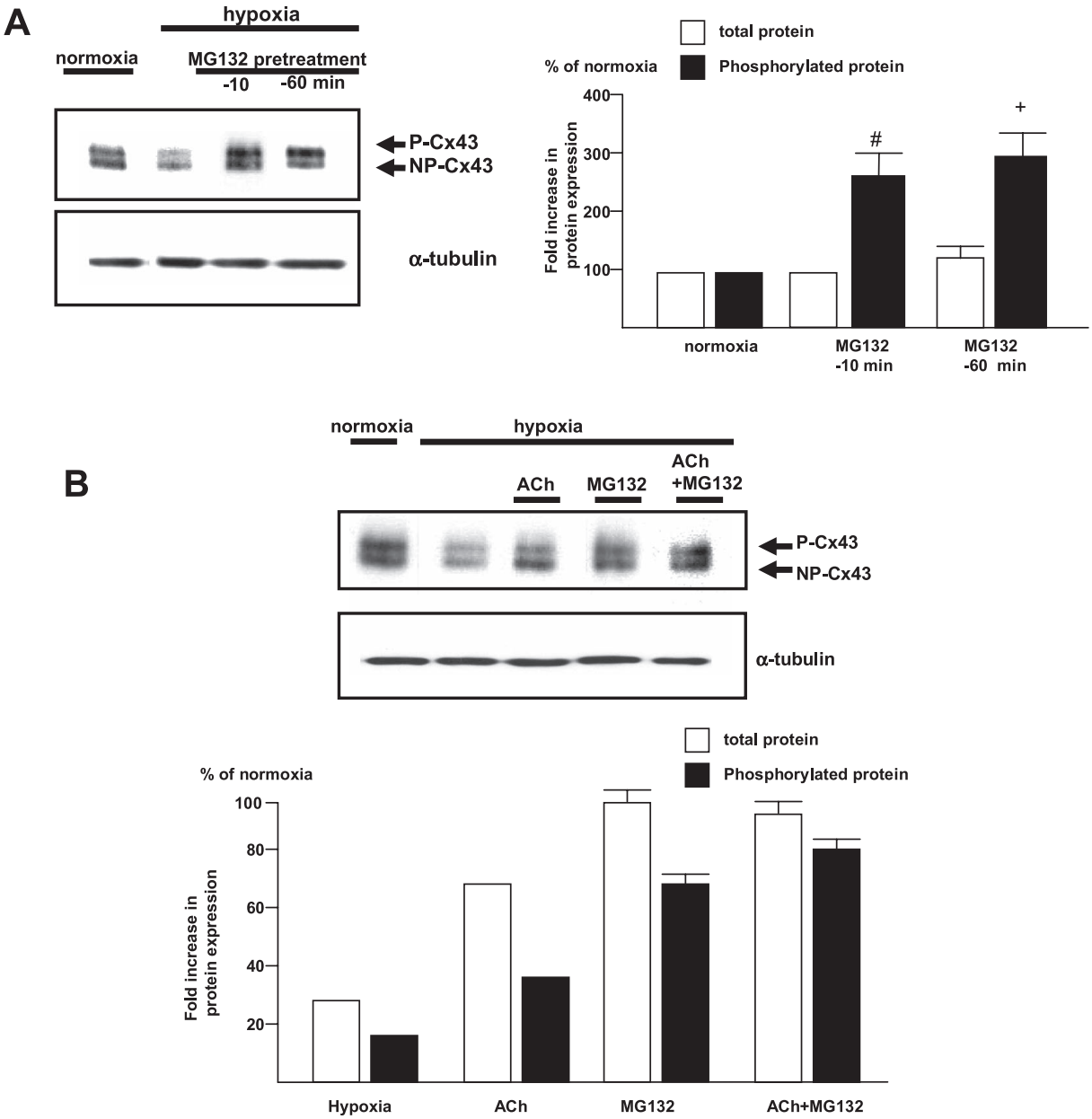

Fig. 4. MG132, a proteasome inhibitor, increases the Cx43 protein level during hypoxia, and cotreatment with ACh does not change this effect. A: Pretreatment with MG132 inhibits the reduction in Cx43 phosphorylation induced by hypoxia ($^{\#}P<0.05$ *vs* normoxia; $^{+}P<0.05$ *vs* normoxia, n = 5). Normoxia: no MG132 or hypoxia. The cells were pretreated with 10 μmol/L MG132 before and at 10 and 60 min of hypoxia. B: The effect of MG132 on inhibiting the reduction in Cx43 phosphorylation is not accentuated by cotreatment with 1 mM ACh since the level of Cx43 phosphorylation with ACh + MG132 is comparable to that with MG132 alone (not significant *vs* MG132, n = 5). Representative data from 5 independently performed experiments are shown (n = 5).

under hypoxia, to the same level observed under normoxia. On the basis of the finding that the survival of Cx43 knockout mice was extremely poor due to ventricular arrhythmia (9, 10), it is suggested that ACh regulates Cx43, which may inhibit arrhythmia.

Our immunohistochemical study supported the result that ACh greatly suppressed the reduction in the Cx43 level under hypoxia. L-NAME inhibited the effect of ACh on Cx43, whereas SNAP mimicked the ACh effect, suggesting that NO is involved in the signaling pathway. ACh is able to induce NO production (17) and has a cardioprotective effect both in vivo and in vitro (18, 19). In fact, H9c2 cells were reported to generate NO from mitochondria in response to ACh (20). Taken together,

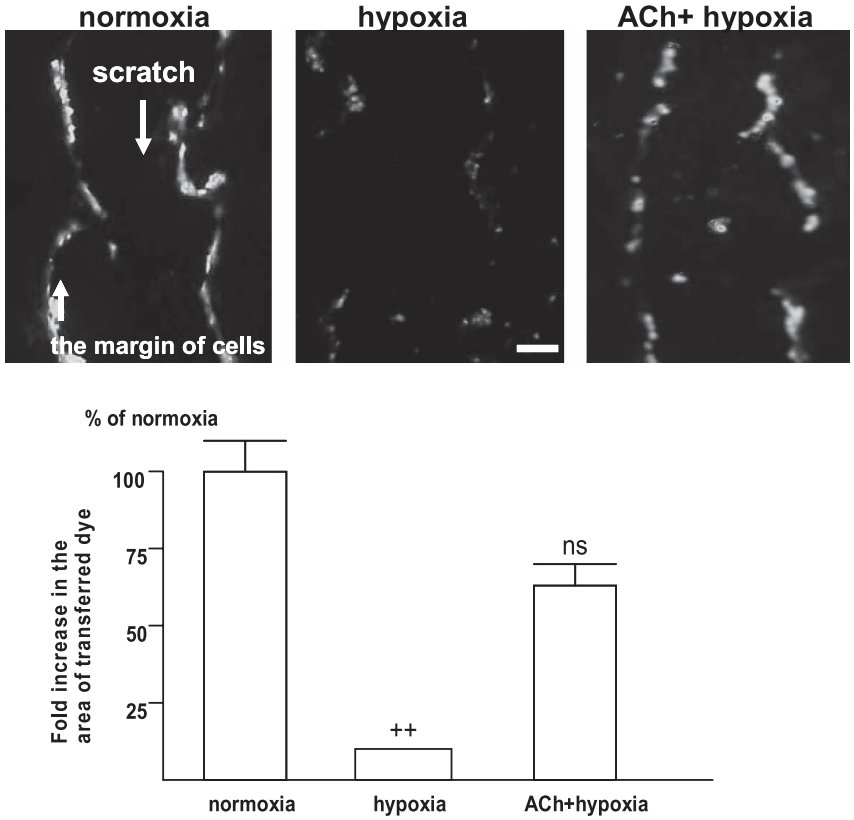

Fig. 5. Fluorescence photomicrographs of scrape/scratch experiments using Lucifer Yellow. Intercellular communication is blocked in H9c2 cells treated with 60 min of hypoxia (hypoxia) ($^{++}P<0.01$ *vs* hypoxia, n = 5). ACh (1 mM) reverses the blockage of intercellular communication induced by hypoxia (ACh + hypoxia) to a comparable level to the control (ns, not significant *vs* normoxia; n = 5) Bar: 150 μm.

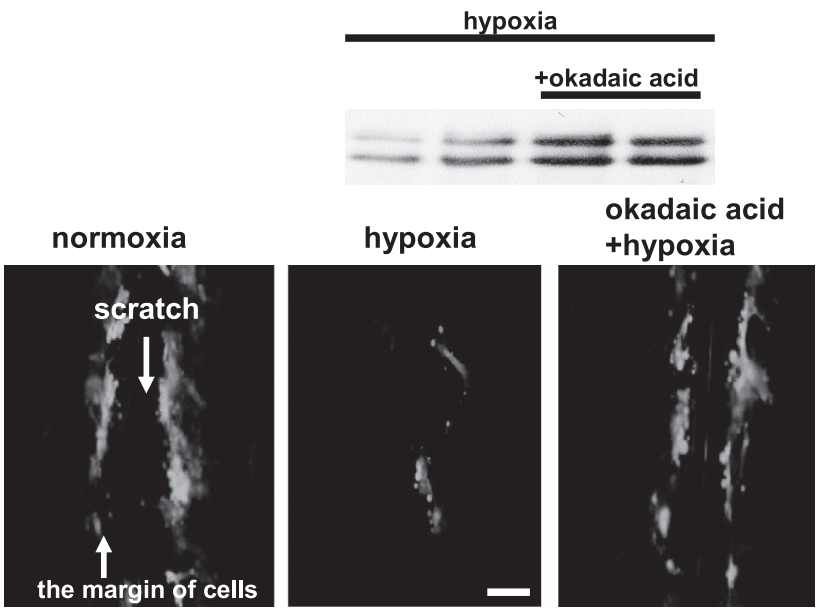

Fig. 6. Phosphatase inhibition recovers cell-cell communication during hypoxia. Pretreatment with okadaic acid (1 μm) for 10 min before hypoxia inhibits the reduction in the Cx43 protein level during hypoxia. Furthermore, it reverses the dye transfer blockage under hypoxia, similar to ACh. Representative data from 3 independently performed experiments are shown (n = 3). Bar: 150 μm.

it is suggested that ACh regulates the Cx43 protein level in cardiomyocytes partly through NO.

To explore whether the Cx43 level during hypoxia was regulated by proteasome degradation, we treated cells with the proteasome inhibitor MG132. Recently, several types of low-molecular-weight proteasome inhibitors have been developed that can readily enter cells and selectively inhibit the protein-degradation pathway. Although their toxicities may sometimes be troublesome experimentally, cell viability and growth are not generally affected by short treatments with these molecules (21 – 23). Surprisingly, MG132 increased the Cx43 protein level, which was reduced under hypoxia, to a comparable level to that after ACh treatment. However, the effect of MG132 on the recovery of Cx43 was not affected by cotreatment with ACh. These results suggest that the proteasome pathway plays a role in Cx43 degradation and that ACh modulates the degradation of Cx43 during hypoxia.

The results of the present study have demonstrated that the increased Cx43 protein level contributes to the functional improvement of gap junctions under hypoxia using the scrape/scratch method. The ACh-induced increase in the Cx43 protein level was functionally involved in the cell-cell communication since ACh recovered Lucifer Yellow transport from the margin of the scratched regions, even under in hypoxia.

As well as the total protein level, the Cx43 phosphorylation level was shown to be involved in its function. Specifically, okadaic acid, a phosphatase inhibitor, recovered the Cx43 protein level and the extent of dye transfer under hypoxia, suggesting that dephosphorylation was partially involved in the hypoxia-induced degradation, eventually leading to a decrease in the total Cx43 protein level. To date, several phosphorylation sites of Cx43 have been reported to have positive or negative effects on gap junctions, suggesting that their function depends on these phosphorylation sites (24). Although we did not investigate the specific phosphorylation site regulated by ACh in the present study, our results suggest that ACh modulates the function of gap junctions through both the protein and phosphorylation levels of Cx43.

Although the scrape/scratch method has some limitations for evaluating cell-cell communication, the result obtained were compatible with those in our previous dye injection study under chemical hypoxia, that is, ACh-treated cardiomyocytes efficiently transferred the dye to surrounding cells, even under hypoxia (4). Therefore, these results suggest that inhibition of the decrease in the Cx43 protein level by ACh under hypoxia is responsible for the enhanced cell-cell communication.

H9c2 cells have been shown to retain several characteristics of the electrical and hormonal signaling pathways found in adult cardiomyocytes and are therefore a useful model for cardiomyocytes from the aspect of signal transduction. The cells show similar morphological characteristics to immature embryonic cardiomyocytes (20).

In conclusion, the results of the present study suggest that ACh activates cell-cell communication by sustaining the Cx43 protein level during hypoxia through modification of the Cx43 degradation pathway.

Acknowledgment

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