

# Nitric Oxide-Mediated Regulation of Connexin43 Expression and Gap Junctional Intercellular Communication in Mesangial Cells

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This study investigated a potential role of nitric oxide (NO) in the regulation of gap junctional intercellular communication (GJIC). Incubation of mesangial cells (MC) with NO donor S-nitroso-N-acetylpenicillamine (SNAP) enhanced both basal and 8-bromo-cAMP-stimulated GJIC as well as expression of gap junction protein connexin43 (Cx43). This potentiating action of SNAP on Cx43 expression was mimicked by two other NO donors and significantly blocked by soluble guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3- $\alpha$ ]quinoxalin-1-1. Guanosine 3',5'-cyclic monophosphate (cGMP) analogue 8-bromo-cGMP exerted an effect similar to NO, whereas another cGMP analogue, 8-pCPT-cGMP, which selectively activates cGMP-dependent kinase without affecting cGMP-inhibited phosphodiesterase (PDE3), had no effect. Moreover, the synergistic action of NO on Cx43 expression was completely prevented by protein kinase A inhibitor H89 but not by cGMP-dependent kinase inhibitor Rp-8-Br-PET-cGMP. These results suggested a possible involvement of NO-cAMP interaction via cGMP-mediated inhibition of PDE3. Indeed, PDE3 inhibitor cilostamide caused potentiation of 8-bromo-cAMP-elicited elevations of Cx43 expression that is similar to the effect of SNAP, and an elevation of intracellular cAMP was detected in SNAP-treated cells. With the use of genetically engineered reporter MC that express secreted alkaline phosphatase under the control of the cAMP response element, significant potentiation of cAMP-elicited activation of cAMP response element by SNAP was found. This effect was abrogated in the presence of PDE3 inhibitor cilostamide. Taken together, the results suggest that NO is involved in the control of GJIC and Cx43 expression. This effect of NO is due to activation of protein kinase A via cGMP-dependent inhibition of PDE3 activity.

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Gap junctions (GJ) are clusters of transmembrane channels that permit the direct intercellular exchange of ions, secondary messengers, and small signaling molecules. Intercellular communication via GJ is thought to play an important role in the control of a variety of cellular functions, including cell growth, migration, differentiation, and electric coupling (1–4). In the cardiovascular system, cells are intensively interlinked by GJ channels (1,4). GJ in vascular cells provide a structural basis for coordinated vasoconstriction or vasodilation and for extensive cross-talk between cells of the vascular wall (1,4). It has been reported that the vasodilative action of the endothelium-derived hyperpolarizing factor (EDHF) is dependent on GJ between endothelial and smooth muscle cells (SMC) (5,6) and that the propagation of vasomotor signals along the vessels also requires GJ intercellular commu-

nication (GJIC) (1,7–9). The altered expression of GJ proteins and its relevance to vascular diseases have also been reported by many investigators (1,4,8,10–12). Therefore, the information on the control of GJ channels in vascular cells is essential for understanding of the role of GJ in vascular pathophysiology.

Nitric oxide (NO) is an important molecule in the vascular system. Besides its vasodilative action, NO exerts multiple effects on SMC, including cell growth, migration, adhesion, and apoptosis. Because GJ channels are known to be important in many cellular functions, it is likely that GJ is involved in the NO-induced alteration of cell behavior. Many effects of NO are caused by induction of a second messenger molecule, guanosine 3',5'-cyclic monophosphate (cGMP), which is produced after NO-mediated activation of soluble guanylate cyclase (sGC). Although cGMP-dependent kinase (PKG) is the major target of intracellular cGMP, cGMP also interacts with other molecules, such as cGMP-gated ion channels. In addition, the effects of cGMP could involve modulation of cAMP levels and protein kinase A (PKA) activity via stimulating phosphodiesterase 2 or via inhibiting phosphodiesterase 3 (PDE3) activity (13–16). High concentrations of cGMP may also directly stimulate PKA (17). cAMP is a well-characterized second mes-

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senger that promotes GJ protein expression and GJIC in a variety of cell types (18,19). We hypothesized that NO may affect GJ protein expression and GJIC via cGMP-mediated cross-talk with cAMP signaling. The purpose of this study was to test this hypothesis.

Mesangial cells (MC) are considered to be specialized SMC that play a pivotal role in the regulation of glomerular hemodynamics. One of the striking features of MC is that these cells possess extremely high density of GJ (20,21). This unique property indicates a possibility that MC are actively involved in the function of the juxtaglomerular apparatus (22). Like SMC, MC mainly express GJ protein connexin 43 (Cx43) (20). We previously reported that the GJ in MC are critically involved in the transmission of intercellular signal and in the coordination of MC contraction (23). However, information is still limited on the regulation of GJ function by pathophysiologic factors.

In this study, we investigated whether and how NO influences GJ protein expression and GJIC in MC. Our present data reveal that NO is a potent stimulator of Cx43 expression and GJIC. We also demonstrate that this action of NO is due to the activation of PKA via cGMP-dependent inhibition of PDE3 activity.

## Materials and Methods

### Materials

cAMP Biotrak enzymeimmunoassay system was purchased from Amersham Biosciences (Buckinghamshire, UK). pCRE-secreted alkaline phosphatase (pCRE-SEAP) vector and Great EscAPe SEAP Detection Kit were obtained from BD Biosciences (Palo Alto, CA). TRITC-conjugated swine anti-rabbit Ig was purchased from DAKO (Glostrup, Denmark), and cilostamide was from Wako (Osaka, Japan). All other reagents were obtained from Sigma (St. Louis, MO).

### MC

Establishment and characterization of rat MC were performed as described previously (23–25).

### Stable Transfection

MC were transfected with pCRE-SEAP (BD Biosciences) together with pcDNA3.1 (Invitrogen, Carlsbad, CA) that encodes a neomycin phosphotransferase gene using a calcium phosphate co-precipitation method (25). Stable transfectants were selected with 0.7 mg/ml G418, and a clone with the lowest background level and the highest SEAP inducibility was selected and used for studies.

### Immunocytochemistry

Immunocytochemical staining for Cx43 was done as previously reported (23,24,26).

### Measurement of GJIC

GJIC was assessed by transfer of the membrane-impermeant fluorescent dye, Lucifer Yellow, after single-cell microinjection with an automated microinjection system (Zeiss Oberkochen, Jena, Germany), as described previously (23,24,26).

### Western Blot Analysis

Equal amounts of cell lysates were separated by 10% SDS-polyacrylamide gels and electrotransferred onto 0.4- $\mu$ M polyvinylidene difluoride membranes. After blocking with 3% BSA in PBS, the membranes

were incubated with anti-Cx43 antibody. After washing with PBS-0.1% Tween 20, filters were probed with horseradish peroxidase-conjugated sheep anti-rabbit IgG or rabbit anti-mouse IgG. Immunoreactivity was detected by the enhanced chemiluminescence system (Amersham Biosciences, Buckinghamshire, UK). For assessing the amount of Cx43 protein loaded, filters were treated with 2% SDS and 100 mM  $\beta$ -mercaptoethanol in 62.5 mM Tris-HCl (pH 6.8) for 30 min at 60°C and reprobed for  $\beta$ -actin. Films were scanned, and the optical density of the bands was measured with Scion Image. Data shown are representative of at least three independent experiments with similar results.

### Northern Blot Analysis

MC were treated with various agents for 12 h. Equal amounts of RNA (5  $\mu$ g) extracted from cells were separated by electrophoresis and transferred onto nylon membranes (Hybond N<sup>+</sup>; Amersham Bioscience). The level of Cx43 mRNA was examined as described before (27), using the entire coding sequence of the rat Cx43 cDNA (28) as a probe (provided by Drs. G. Olbina and W. Eckhart, Molecular and Cell Biology Laboratory, The Salk Institute for Biologic Studies, San Diego, CA). The staining of 28S and 18S ribosomal RNA by ethidium bromide was used for loading controls.

### cAMP Measurement

Confluent MC were exposed to 100  $\mu$ M S-nitroso-N-acetylpenicillamine (SNAP) or left untreated for 30 min. After the stimulation, the cells were lysed and assayed for cAMP according to the manual instruction for cAMP Biotrak Enzymeimmunoassay System. A part of cellular lysate was used for protein assay using Micro BCA Protein Assay Kit (Pierce, Rockford, IL).

### SEAP Assay

Activity of SEAP was evaluated using the Great EscAPe Detection Kit following the protocol provided by the manufacturer. In brief, MC in 96-well plates were exposed to various stimuli for 24 h. Aliquots of supernatants were sampled from the cultures and centrifuged at 12,000  $\times$  g for 2 min. Fifteen microliters of dilution buffer was mixed with 5  $\mu$ l of sample, and the mixture was incubated at 65°C for 30 min to eliminate the endogenous alkaline phosphatase activity. Twenty microliters of assay buffer was subsequently added to the mixture and incubated for an additional 10 min at room temperature. The CSPD substrate at a concentration of 1.25 mM was prepared by dilution with 20 $\times$  chemiluminescence enhancer, and 20  $\mu$ l of the diluted substrate was added to each sample, followed by 10 min at room temperature. The intensity of chemiluminescent signal was determined by a luminometer (Gene Light 55; Microtech Niton, Chiba, Japan).

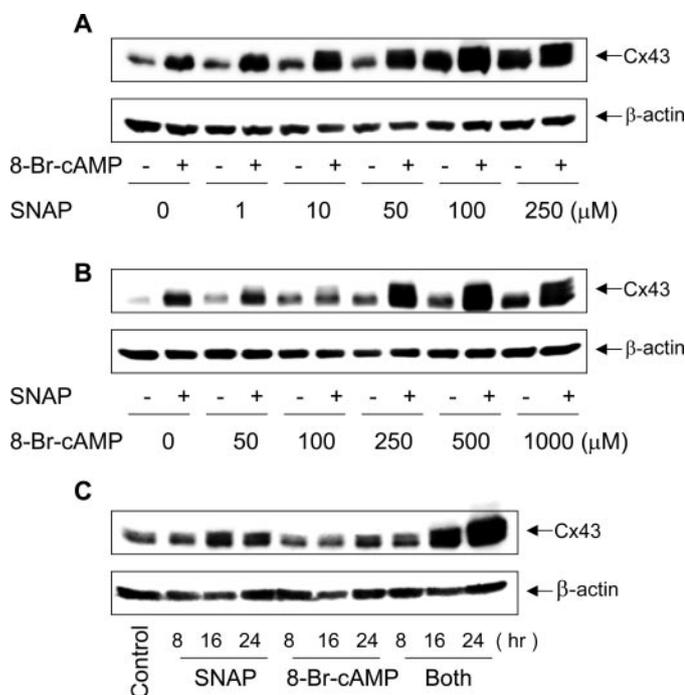
### Statistical Analyses

Values are expressed as either means  $\pm$  SEM or means  $\pm$  SD. Comparison with control was done with one-way ANOVA and the Dunnett test. Comparison of two populations was made by *t* test. *P* < 0.05 was considered to be statistically significant.

## Results

### NO Induces Cx43 Expression

Incubation of MC with NO donor SNAP for 24 h resulted in a dose-dependent increment of Cx43 protein (Figure 1A). This effect of SNAP was much more enhanced in the presence of a cell-permeable analogue of cAMP, 8-bromo-cAMP (500  $\mu$ M). The enhancement of Cx43 expression by SNAP was observed at a wide range of concentrations from 1 to 250  $\mu$ M. 8-Bromo-



**Figure 1.** Effects of nitric oxide (NO) donor S-nitroso-N-acetylpenicillamine (SNAP) on basal and cAMP-elicited expression of connexin43 (Cx43). (A) Dose-dependent induction of Cx43 expression by SNAP. Rat mesangial cells (MC) were exposed to various concentrations of SNAP in the presence (+) or absence (–) of 500  $\mu$ M 8-bromo-cAMP for 24 h. Cellular protein was extracted and subjected to Western blot analysis using either an anti-Cx43 antibody (top row) or an anti- $\beta$ -actin antibody (bottom row). (B) Potentiation of 8-bromo-cAMP-elicited expression of Cx43 by SNAP. MC were treated for 24 h with various concentrations of 8-bromo-cAMP in the presence or absence of SNAP (100  $\mu$ M), and expression of Cx43 was evaluated. (C) Time-dependent effects of SNAP and 8-bromo-cAMP on Cx43 expression. MC were treated with 100  $\mu$ M SNAP, 250  $\mu$ M 8-bromo-cAMP, or both for indicated durations, and Cx43 expression was examined.

cAMP also induced a dose-dependent elevation of Cx43, and its effect was dramatically potentiated when it was added together with 100  $\mu$ M SNAP (Figure 1B). A time-course analysis revealed that the marked elevation of Cx43 occurred within 16 h after exposure to these stimuli (Figure 1C).

The action of SNAP on Cx43 expression was mimicked by other structurally unrelated NO donors, (Z)-1-[2-(aminoethyl)-N-(2-ammonioethyl)amino]diazene-1-ium-1,2-diolate (DETA-NONOate) and sodium nitroprusside (SNP; Figure 2A), suggesting that the common product of these agents, NO but not other metabolites, was responsible for the elevated expression of Cx43.

#### Effect of NO on Cx43 Expression Is Mediated not by PKG but by PKA

Most of the biologic actions of NO are supposed to be mediated by cGMP signals (16). We therefore examined the role of the cGMP pathway in Cx43 expression. First, we tested whether

an inhibitor of sGC, 1H-[1,2,4]oxadiazolo[4,3,- $\alpha$ ]quinoxalin-1-1 (ODQ), diminishes the effect of NO on Cx43 expression. As shown in Figure 2A, ODQ at 25  $\mu$ M unexpectedly increased the basal level of Cx43 for reasons presently unknown. Even so, ODQ substantially prevented the potentiating actions of three different NO donors (SNAP, NONOate, and SNP). Second, we tested whether the stable, lipophilic analogues of cGMP (8-bromo-cGMP and 8-pCPT-cGMP) can mimic the promoting action of SNAP on Cx43 expression. As demonstrated in Figure 2B, 8-bromo-cGMP but not 8-pCPT-cGMP (which selectively activates PKG but does not affect cGMP-inhibited phosphodiesterase) exerted the effect similar to SNAP on Cx43. Of note, both agents at the same concentration used (500  $\mu$ M) elicited comparable activation of PKG, as evaluated by the phosphorylation levels of vasodilator-stimulated phosphoprotein at serine 239 (a site preferentially phosphorylated by PKG) (29) (data not shown). Thus, it is unlikely that the potentiating action of SNAP on Cx43 expression was mediated by PKG activation. Indeed, pretreatment of cells with PKG-specific inhibitor Rp-8-Br-PET-cGMP (50  $\mu$ M) did not attenuate the effect of SNAP (Figure 2B).

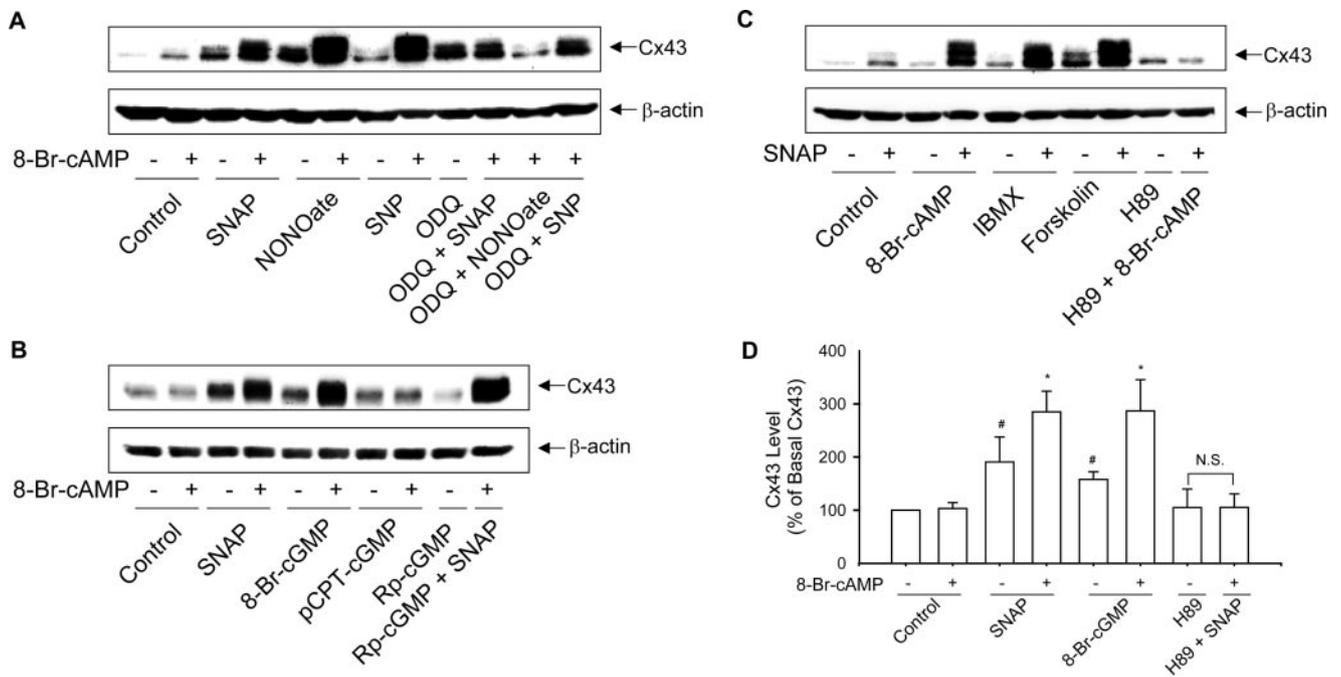
Given that cAMP is a well-known second messenger in promoting both Cx43 expression and GJIC (14,18,19), a role of the cAMP signaling in the action of NO was examined. We first tested whether SNAP can enhance Cx43 expression triggered by cAMP-elevating agents other than 8-bromo-cAMP. As shown in Figure 2C, SNAP (100  $\mu$ M) significantly potentiated the expression of Cx43 triggered by low concentrations of 3-isobutyl-1-methylxanthine (a nonspecific PDE inhibitor; 5  $\mu$ M) and forskolin (a direct activator of adenylyl cyclase; 1  $\mu$ M). Because cAMP-dependent effects are usually mediated by PKA, we further investigated the role of PKA in the expression of Cx43. As demonstrated in Figure 2C, pretreatment of cells with PKA inhibitor H89 (10  $\mu$ M) completely blocked the synergistic effect of SNAP and 8-bromo-cAMP on Cx43 expression.

The densitometric analysis of the main findings shown in Figures 1 and 2 is summarized in Figure 2D. NO donor SNAP significantly enhanced both basal and 8-Br-cAMP-elicited Cx43 expression. This action of SNAP was mimicked by 8-Br-cGMP and completely blocked by PKA inhibitor H89.

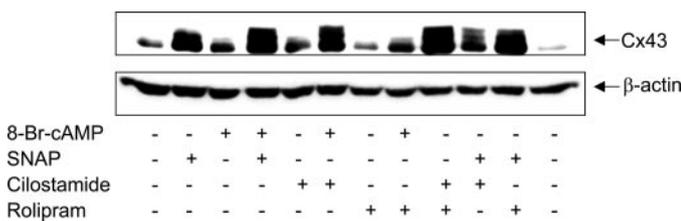
It should be noted that treatment of cells with NO and 8-bromo-cAMP not only increased the amount of Cx43 but also altered the status of Cx43 phosphorylation. In control cells, mainly nonphosphorylated Cx43 was observed. It appeared as one band in Western blots, whereas in the treated cells, Cx43 existed in both phosphorylated and nonphosphorylated forms, which appeared as double or triple bands in Western blot. The slower migrating band is considered to correspond to a phosphorylated form of Cx43.

#### PDE3 Inhibitor Mimics the Effect of NO

One possible mechanism by which NO stimulates PKA activation is an increase in cAMP concentration caused by cGMP-mediated inhibition of PDE3 (13–15). To examine this possibility, we compared the effect of SNAP with that of a specific PDE3 inhibitor, cilostamide. As shown in Figure 3, treatment of cilostamide (20  $\mu$ M) increased Cx43 expression. In combination



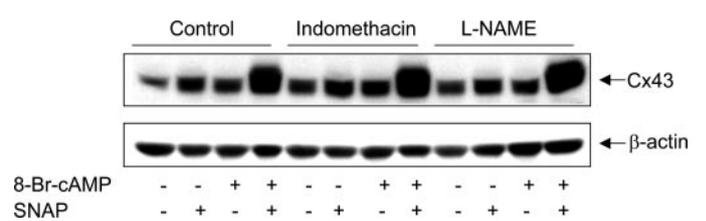
**Figure 2.** Effects of various inhibitors on the action of SNAP. (A) Prevention of the potentiating effects of NO donors by 1H-[1,2,4]oxadiazolo[4,3- $\alpha$ ]quinoxalin-1-1 (ODQ). MC were treated either with or without 25  $\mu$ M ODQ for 15 min and exposed to three different NO donors [100  $\mu$ M SNAP, 100  $\mu$ M (Z)-1-[2-(aminoethyl)-N-(2-ammonioethyl)amino]diazene-1-ium-1,2-diolate (NONOate) and 200  $\mu$ M sodium nitroprusside (SNP)] together with 250  $\mu$ M 8-bromo-cAMP for 24 h. The levels of Cx43 and  $\beta$ -actin were determined by Western blot analysis. (B) Lack of involvement of guanosine 3',5'-cyclic monophosphate (cGMP)-dependent kinase (PKG) in the effect of SNAP. MC were treated with SNAP (100  $\mu$ M), 8-bromo-cGMP (500  $\mu$ M), or 8-pCPT-cGMP (500  $\mu$ M) alone or in combination with 8-bromo-cAMP (250  $\mu$ M) for 24 h. For analyzing a role of PKG, cells were pretreated with a specific PKG inhibitor, Rp-8-Br-PET-cGMP (Rp-cGMP; 50  $\mu$ M) for 15 min and then exposed to SNAP and 8-bromo-cAMP for 24 h in the presence of the PKG inhibitor. (C) Blockade of the potentiating effect of SNAP on Cx43 expression by protein kinase A (PKA) inhibitor. MC were treated with various cAMP-elevating agents (250  $\mu$ M 8-bromo-cAMP; 5  $\mu$ M 3-isobutyl-1-methylxanthine [IBMX] or 1  $\mu$ M forskolin) alone or in combination with 100  $\mu$ M SNAP for 24 h. For examining the role of PKA, cells were pretreated with PKA inhibitor H89 (10  $\mu$ M) for 15 min before the exposure of SNAP plus 8-bromo-cAMP for 24 h with the PKA inhibitor. (D) Densitometric analysis of the main data shown in A, B, and C. The results are expressed as percentages of the basal Cx43 (mean  $\pm$  SEM,  $n = 3$  to 5). # $P < 0.01$  versus basal control; \* $P < 0.01$  versus single stimulus.



**Figure 3.** Effect of phosphodiesterase (PDE) inhibitors on Cx43 expression. MC were treated with 8-bromo-cAMP (250  $\mu$ M), SNAP (100  $\mu$ M), cilostamide (20  $\mu$ M), or rolipram (20  $\mu$ M) alone or in combination for 24 h. Expression of Cx43 was examined by Western blot analysis. Similar results were obtained by two additional experiments.

with 250  $\mu$ M 8-bromo-cAMP, a synergistic effect was observed. In contrast, PDE4 inhibitor rolipram (20  $\mu$ M) had no effect on the expression of Cx43.

Several previous studies have documented that PDE3 and PDE4 inhibitors work synergistically in modulating multiple cell behavior (30,31). We tested whether these agents also act



**Figure 4.** Influences of cyclooxygenase or NO synthase inhibitor on the synergistic action of SNAP and 8-bromo-cAMP. MC were either left untreated (control) or treated with 50  $\mu$ M indomethacin or 100  $\mu$ M N<sup>G</sup>-nitro-L-arginine methyl ester 15 min before exposure to SNAP (100  $\mu$ M) and 8-bromo-cAMP (500  $\mu$ M) alone or in combination for 24 h. Expression of Cx43 was examined by Western blot analysis. Similar results were obtained by two additional experiments.

synergistically in inducing Cx43 expression. As shown in Figure 3, the combination of PDE3 and PDE4 inhibitors markedly induced the expression of Cx43. A similar synergy was also observed between SNAP and the PDE4 inhibitor rolipram.

These observations indicated a possibility that the potentiating action of SNAP on Cx43 expression is due to inhibition of PDE3 activity.

#### Cx43 Expression Was not Altered by Cyclooxygenase or NO Synthase Inhibitor

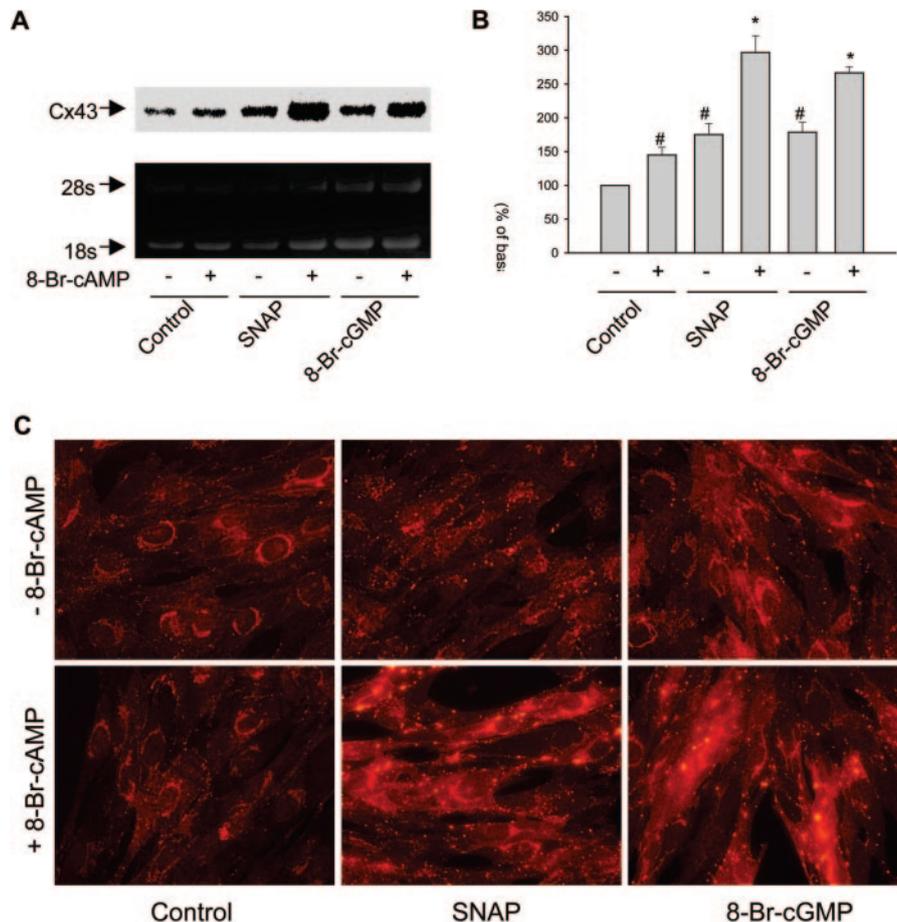
cAMP signaling pathway and NO have extensive cross-talk. Besides inhibition of PDE3, NO is reported to be able to induce and activate the cyclooxygenase (32,33). The increased formation of prostaglandin E<sub>2</sub>, a known activator of the adenylyl cyclase, may contribute to the action of NO on Cx43 expression. In addition, cAMP has been reported to trigger the expression of inducible NO synthase in MC (34). The positive reciprocal interactions between endogenous NO and cAMP may also be implicated in the control of Cx43 expression. To examine these possibilities, we pretreated MC with the specific cyclooxygenase inhibitor indomethacin or with the NO synthase inhibitor N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME) before exposing the cells to NO, 8-Br-cAMP alone, or both in combination.

Figure 4 shows that neither indomethacin nor L-NAME affected the expression of Cx43 as induced by the stimuli. Therefore, these possibilities were excluded.

#### NO Increases Cx43 mRNA Expression and Membrane Location of Cx43 Protein

To examine whether NO affects Cx43 expression at the transcriptional level, we performed Northern blot analysis. Consistent with the increase in the protein level, SNAP significantly induced mRNA expression of Cx43 under both basal and cAMP-stimulated conditions. This effect of NO could also be mimicked by 8-bromo-cGMP (Figure 5, A and B).

Cx43 is known to be located at cell-to-cell contact portions and perinuclear regions in cultured MC (23), and only membrane-associated Cx43 molecules are active for GJIC. We looked at effects of SNAP and 8-bromo-cGMP on Cx43 protein distribution by using immunofluorescent staining. As shown in Figure 5C, Cx43 was mainly located at the perinuclear regions in untreated controls. After exposure to SNAP (100  $\mu$ M) or 8-bro-

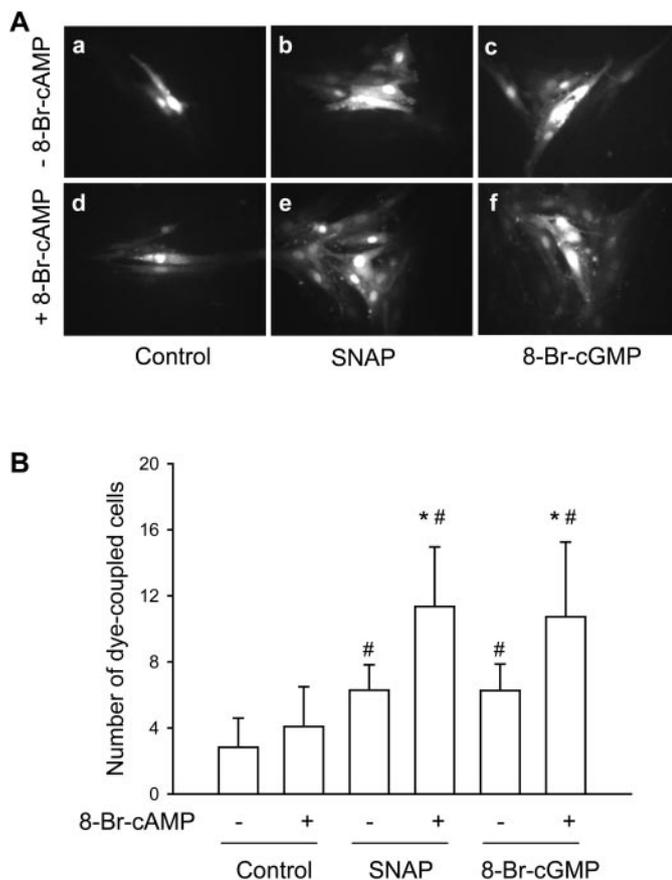


**Figure 5.** Effects of NO on mRNA expression and protein distribution of Cx43. (A) Northern blot analysis of Cx43 mRNA expression in MC that were treated with 100  $\mu$ M SNAP and 500  $\mu$ M 8-bromo-cGMP alone or in combination with 250  $\mu$ M 8-bromo-cAMP for 12 h (top). Staining of ribosomal RNA 18S and 28S was shown as a loading control (bottom). One representative result from three independent experiments is shown. (B) Densitometric analysis of data shown in A. Data are expressed as relative percentages against the value of untreated control (means  $\pm$  SEM;  $n = 3$ ). # $P < 0.01$  versus basal control; \* $P < 0.01$  versus single stimulus. (C) MC were treated with 100  $\mu$ M SNAP or 500  $\mu$ M 8-bromo-cGMP alone (Figure 4C, top) or in combination with 250  $\mu$ M 8-bromo-cAMP (Figure 4, bottom) for 24 h and subjected to immunofluorescent staining of Cx43. Magnification,  $\times 600$ .

mo-cGMP (500  $\mu$ M) for 24 h, a modest but clear enhancement of Cx43 staining was observed along the region of cell-to-cell contact. When SNAP or 8-bromo-cGMP was added in combination with 8-bromo-cAMP, a dramatic increase of Cx43 was observed at both the cell-to-cell contact region and the perinuclear region. 8-Bromo-cAMP alone had only a marginal effect on Cx43 staining at the concentration of 250  $\mu$ M.

### NO Augments Functional GJIC

The increased expression and membrane distribution of Cx43 may lead to enhancement of GJIC. To examine this possibility, we analyzed the GJIC in MC using the Lucifer Yellow dye-transfer assay (23,24,26). As shown in Figure 6, incubation of MC with 100  $\mu$ M SNAP or 500  $\mu$ M cGMP was found to be sufficient for promotion of GJIC (number of dye-coupled cells: control,  $2.83 \pm 1.75$ ; SNAP,  $6.28 \pm 1.54$ ; 8-bromo-cGMP,  $6.26 \pm$



**Figure 6.** Promotion of gap junctional intercellular communication (GJIC) by NO. (A) Microphotographic analysis of dye-coupled cells. Confluent culture of MC was treated with SNAP (100  $\mu$ M) or 8-bromo-cGMP (500  $\mu$ M) together with (+) or without (-) 8-bromo-cAMP (250  $\mu$ M) for 24 h, and Lucifer Yellow (LY) was pressure-injected into a single cell. LY diffusion into adjacent cells was recorded by video camera. (B) Quantitative analysis of dye-coupled cells. The number of cells into which LY was transferred from the injected cells within 3 min is shown. Data are presented as the means  $\pm$  SEM ( $n = 10$  to 15). # $P < 0.01$  versus basal control; \* $P < 0.01$  versus single stimulus. Magnification,  $\times 320$  in A.

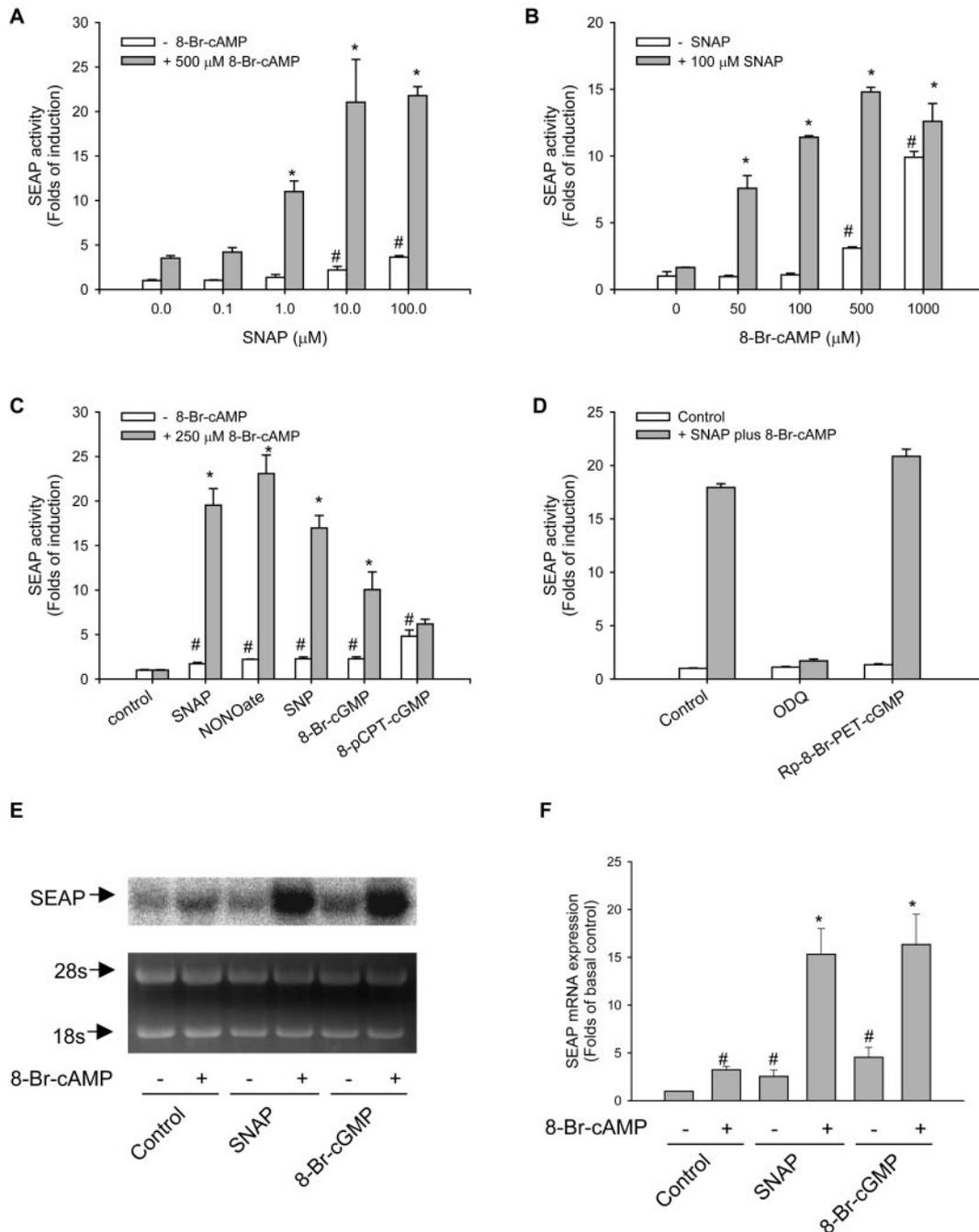
1.59; means  $\pm$  SEM;  $n = 10$  to 15). In the presence of 250  $\mu$ M 8-bromo-cAMP, the effects of SNAP and 8-bromo-cGMP were more pronounced (8-bromo-cAMP alone,  $4.09 \pm 2.40$ ; cAMP + SNAP,  $11.35 \pm 3.61$ ; cAMP + 8-bromo-cGMP,  $10.73 \pm 4.52$ ; means  $\pm$  SEM;  $n = 10$  to 15).

### Amplification of the cAMP Signaling by NO

To provide direct evidence showing that NO is able to amplify the cAMP signaling, the level of intracellular cAMP was measured. Significant elevation of the cAMP level was detected in cells that were treated with 100  $\mu$ M SNAP for 30 min (cAMP concentration [fmol/L per  $\mu$ g protein]: basal,  $6.29 \pm 1.47$  versus 100  $\mu$ M SNAP,  $10.84 \pm 1.98$ ; mean  $\pm$  SEM;  $n = 3$ ;  $P < 0.05$ ).

The increase of cAMP causes PKA activation. It subsequently activates cAMP response element (CRE), leading to expression of genes that have CRE in their regulatory regions. Because the promoter region of the Cx43 gene contains CRE (35) and that CRE activity serves as a good indicator of PKA activation, we examined activity of CRE in MC that were treated with SNAP. For this purpose, MC were stably transfected with pCRE-SEAP, and reporter cells were established. The cells were then exposed to various stimuli, and activity of SEAP in conditioned media was evaluated. As shown in Figure 7A, SNAP alone induced a modest increase of CRE activity in a dose-dependent manner. The activation of CRE by SNAP was dramatically enhanced in the presence of 500  $\mu$ M 8-bromo-cAMP. Figure 7B shows enhancement of 8-bromo-cAMP-induced activation of CRE by SNAP. This effect was observed at a wide range of concentrations of 8-bromo-cAMP (50 to 500  $\mu$ M). Similar to our previous data on Cx43 expression, the potentiating effect of SNAP on CRE activity was mimicked by other NO donors NONOate and SNP and by cGMP analogue 8-bromo-cGMP but not by 8-pCPT-cGMP (Figure 7C). It is interesting, however, that 8-pCPT-cGMP alone induced a higher basal level of CRE activity, as compared with SNAP or 8-bromo-cGMP. Consistent with the results shown in Figure 2A, the effect of SNAP on CRE activation was prevented by ODQ but not by PKG inhibitor Rp-8-Br-PET-cGMP (Figure 7D). The synergistic effect of SNAP or 8-bromo-cGMP on cAMP-elicited activation of CRE was also confirmed by the mRNA level of the reporter gene SEAP (Figure 7, E and F).

Consistent with the critical role of PDE3 inhibition in the action of SNAP, PDE3 inhibitor cilostamide was found to mimic the potentiating action of SNAP on the cAMP-elicited activation of CRE. Furthermore, in the presence of cilostamide, SNAP failed to exhibit any additional effects on CRE activity (Figure 8A), suggesting that both agents may function through the same mechanism. Consistent with the previous data showing the synergistic effect between PDE3 and PDE4 inhibitors on Cx43 expression, a similar, additive effect between these agents on CRE activation was observed (Figure 8B). The role of PDE3 inhibitor cilostamide in this cooperative action was completely reproduced by SNAP (Figure 8B). In other words, SNAP acted like the PDE3 inhibitor cilostamide in modulating cAMP signaling.



**Figure 7.** Potentiating action of NO on cAMP response element (CRE) activity. (A) Dose-dependent potentiation of 8-bromo-cAMP-elicited CRE activation by SNAP. MC that were stably transfected with pCRE–secreted alkaline phosphatase (pCRE–SEAP) were exposed to the indicated concentrations of SNAP in the presence or absence of 500  $\mu\text{M}$  8-bromo-cAMP for 24 h. The conditioned media were harvested and assayed for SEAP activity. (B) Enhancement of the action of 8-bromo-cAMP by SNAP. MC were treated with the indicated concentrations of 8-bromo-cAMP alone or together with 100  $\mu\text{M}$  SNAP for 24 h. (C) Various NO donors and cGMP analogues on CRE activity. MC were treated with 100  $\mu\text{M}$  SNAP, 100  $\mu\text{M}$  NONOate, 200  $\mu\text{M}$  SNP, 500  $\mu\text{M}$  8-bromo-cGMP, or 500  $\mu\text{M}$  8-pCPT-cGMP alone or in combination with 250  $\mu\text{M}$  8-bromo-cAMP for 24 h. (D) Effects of soluble guanylate cyclase (sGC) inhibitor ODQ and PKG inhibitor Rp-8-Br-PET-cGMP on the action of NO. MC were preincubated with 25  $\mu\text{M}$  ODQ or 50  $\mu\text{M}$  PKG inhibitor Rp-8-Br-PET-cGMP for 15 min before exposing to the mixture of SNAP (100  $\mu\text{M}$ ) and 8-bromo-cAMP (250  $\mu\text{M}$ ) for 24 h in the presence of these agents. Data are presented as fold induction of SEAP activity against untreated control (means  $\pm$  SD). Assays were performed in triplicate. # $P$  < 0.05 versus basal control; \* $P$  < 0.05 versus single stimulus. Similar results were obtained from an additional experiment. (E) Northern blot analysis of SEAP mRNA expression in reporter cells that were treated with SNAP or 8-bromo-cGMP alone or in combination with 8-bromo-cAMP for 12 h (top). The staining of ribosomal RNA 18S and 28S is shown in the bottom panel as a loading control. Representative results from three independent experiments are shown. (F) Densitometric analysis of data shown in E. The results are expressed as fold induction against the untreated control (means  $\pm$  SEM;  $n$  = 3). # $P$  < 0.01 versus basal control; \* $P$  < 0.01 versus single stimulus.

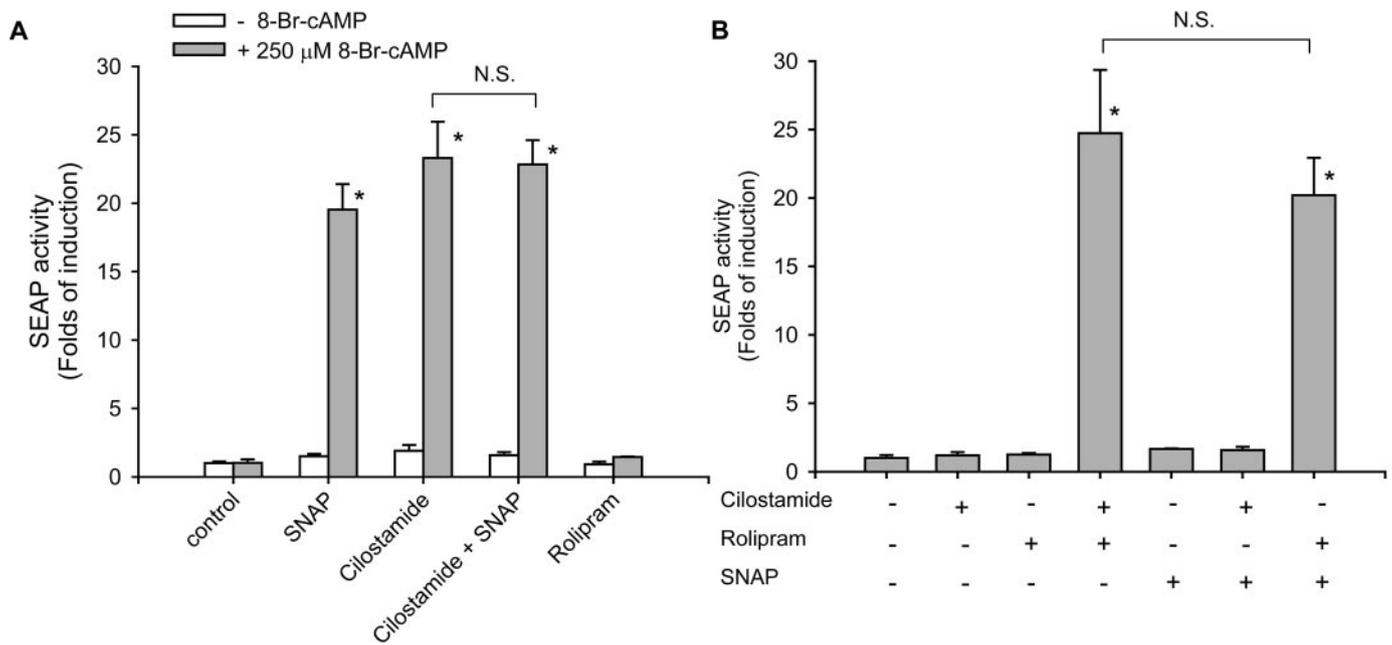


Figure 8. Reproduction of the action of SNAP on CRE activity by PDE3 inhibitor. The CRE reporter MC were treated with 8-bromo-cAMP (250 μM), SNAP (100 μM), cilostamide (20 μM), or rolipram (20 μM) alone or in various combinations for 24 h. Conditioned media were collected and used for SEAP assay. Data are presented as fold induction (means ± SD) of SEAP activity against untreated control. Assays were performed in triplicate. Similar results were also obtained from at least one additional experiment. \**P* < 0.05 versus single stimulus.

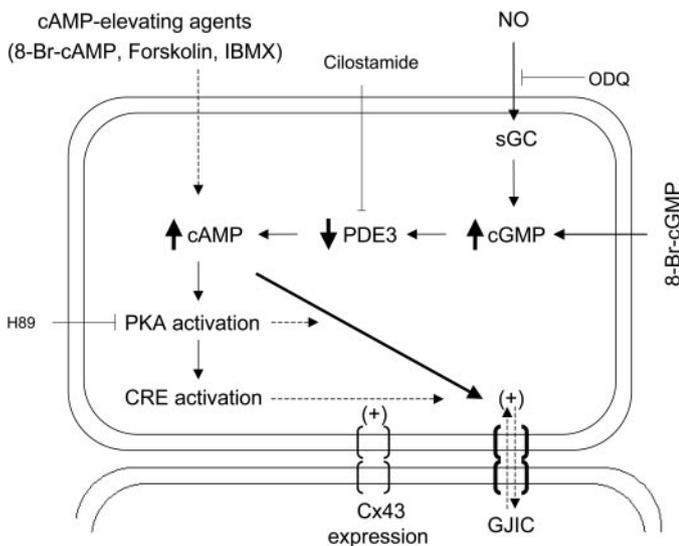
### Discussion

In this study, we investigated the effects of NO on Cx43 expression and GJIC in cultured MC. Using NO donor SNAP as an exogenous source of NO, we demonstrated that NO markedly increased basal and cAMP-stimulated levels of Cx43 expression, leading to enhanced GJIC. The mechanism underlying this effect involved activation of sGC, elevation of cGMP, and amplification of the cAMP signaling via the cGMP-mediated inhibition of PDE3. Our current hypothesis is illustrated in Figure 9.

In this report, we showed, for the first time, that NO is able to enhance both basal and cAMP-elicited Cx43 expression and GJIC. The effect of NO donor on Cx43 expression was detected at several different levels; *i.e.*, the protein level detected by immunofluorescent staining and Western blot, the transcriptional level detected by Northern blot, and the functional level examined by the dye-transfer assay. The regulation of GJ by NO has been reported previously in some cell types. Sladek *et al.* (36) showed that endogenous NO attenuated myometrial Cx43 expression during rat pregnancy. Another report, by Roh *et al.* (37), also demonstrated an inhibitory effect of exogenous NO on Cx43 levels in cultured uterine myocytes. The mechanisms involved are currently unknown. The discrepancy of the results from uterine myocytes and glomerular MC may be due to the different properties of the cells used for study. Contrary to most of the previous observations (18,19), cAMP has been shown to inhibit Cx43 expression in myocytes (37). In a recent study using human umbilical vein endothelial cells, NO was shown to enhance endothelial cell coupling and selectively increase the expression of Cx40 but not Cx43 (12). However,

information is very limited regarding how NO affects Cx43 expression and cell coupling in vascular effector cells. Because Cx43 is the predominant GJ protein in SMC and plays a critical role in transmission of vasomotor signals as well as in coordination of vascular responses (1,4), regulation of Cx43 expression and GJIC by the major endothelium-derived vasorelaxing factor NO should have significant pathophysiologic relevance.

We demonstrated that the effects of the NO donors were significantly attenuated by sGC inhibitor ODQ. This inhibitory effect points to a possible role of cGMP in the NO-mediated regulation of Cx43 expression and GJIC. Indeed, a stable analogue of cGMP, 8-bromo-cGMP, completely mimicked the ability of NO to potentiate Cx43 expression and GJIC. cGMP-dependent actions of NO are usually mediated by PKG (16). However, in our experimental setting, blockade of PKG with specific PKG inhibitor Rp-8-bromo-PET-cGMP did not influence the promoting effect of NO on Cx43 expression. In addition, the selective PKG agonist 8-pCPT-cGMP did not mimic the action of NO. The PKG-independent induction of Cx43 by NO led us to explore alternative responsible pathways. Because cAMP is the well-characterized second messenger that promotes GJIC and Cx43 expression (18,19), we investigated the relationship between NO and cAMP signaling. Our data demonstrated that the action of NO was totally prevented by PKA inhibitor H89. The cGMP-mediated activation of PKA is caused by either a direct effect of cGMP on PKA (17) or an indirect action of cGMP via inhibition of cGMP-inhibited cAMP-hydrolyzing PDE3 that increases the level of cAMP and activates PKA (15). Our findings support the later possibility, because (1) PDE3 inhibitor mimicked the action of NO in potentiating



**Figure 9.** Schematic diagram illustrating potential mechanisms involved in the NO-mediated regulation of Cx43 expression and GJIC. NO activates sGC, causing the generation and action of cGMP. The cGMP-dependent inhibition of PDE3 activity results in the increase of the cAMP level and subsequent activation of PKA, leading to the enhancement of Cx43 expression and GJIC.

cAMP-elicited Cx43 expression; (2) increased levels of cAMP were detected after exposure of the cells to SNAP; (3) cGMP analogue 8-pCPT-cGMP, which selectively activates PKG but does not interact with PDE3, had no obvious effect; and (4) in the presence of PDE3 inhibitor, the potentiating action of SNAP on CRE activation could not be observed. Taken together, our current data support the idea that the PDE3 inhibition-mediated increase of cAMP and subsequent activation of PKA is the major mechanism for SNAP-mediated potentiation of Cx43 expression and GJIC (Figure 9).

The findings described in this report may have significant pathophysiologic implications as follows: (1) because GJ channels are known to be important in many cellular functions, modulation of GJ might be one of the mechanisms by which NO regulates cell behavior; and (2) besides NO, endothelium-derived vasorelaxing factors, including prostacyclin and EDHF, exert their biologic effects via the cAMP pathway (5). NO may facilitate their vasodilative function via amplification of the cAMP signaling. This synergistic action of NO may be especially important for EDHF, because its function is presumably mediated by GJIC (5,6). Furthermore, a positive reciprocal interaction between intracellular cAMP and EDHF vasorelaxing activity has been documented recently (3,5). The co-treatment of cells with PDE3 and PDE4 inhibitors induced dramatic activation of CRE and remarkable upregulation of Cx43. This finding may lead to the development of a novel strategy for therapeutic intervention in diseases in which impaired GJIC is involved. Activation of CRE and upregulation of Cx43 expression may be the important underlying pharmacologic mechanisms of their actions under a variety of conditions (30,31).

In summary, our study demonstrated that NO is a potent

modulator of Cx43 expression and GJIC. This finding may open a new window toward our further understanding of the vascular role of NO under various pathophysiologic situations.

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