Reciprocal Regulation between Proinflammatory Cytokine-induced Inducible NO Synthase (iNOS) and Connexin43 in Bladder Smooth Muscle Cells

Kai Li,†‡§, Jian Yao,† Liye Shi,† Norifumi Sawada,† Yuan Chi,§ Qiaojing Yan,§ Hiroyuki Matsue‡*, Masanori Kitamura,§ and Masayuki Takeda†

From the Departments of †Molecular Signaling and §Urology, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, Chuo, Yamanashi 409-3898, Japan, ‡Department of Dermatology, Graduate School of Medicine, Chiba University, Chiba 260-8670, Japan, and the Departments of §Oncology and §Cardiology, First Affiliated Hospital, China Medical University, Shenyang 110001, China

Gap junctions (GJs) play an important role in the control of bladder contractile response and in the regulation of various immune inflammatory processes. Here, we investigated the possible interaction between inflammation and GJs in bladder smooth muscle cells (BSMCs). Stimulation of BSMCs with IL1β and TNFα increased connexin43 (Cx43) expression and function, which was associated with increased phosphorylation of vasodilator-stimulated phosphoprotein. Inhibition of PKA with H89 or down-regulation of CREB with specific siRNAs largely abolished the Cx43-elevating effect. Further analysis revealed that IL1β/TNFα-induced NFκB-dependent inducible NO synthase (iNOS) expression. Inhibition of iNOS with G-nitro-L-arginine methyl ester abrogated and an exogenous NO donor mimicked the effect of the cytokines on Cx43. Intraperitoneal injection of LPS into mice also induced bladder Cx43 expression, which was largely blocked by an iNOS inhibitor. Finally, the elevated Cx43 was found to negatively regulate iNOS expression. Dysfunction of GJs with various blockers or down-regulation of Cx43 with siRNA significantly potentiated the expression of iNOS. Fibroblasts from Cx43 knockout (Cx43−/−) mice also displayed a significantly higher response to the cytokine-stimulated Cx43. The results suggest that IL1β/TNFα has a positive impact on Cx43 expression. In contrast, the increased NO production may negatively regulate Cx43 expression.

Acute cystitis is a common disease afflicting millions of people each year and is usually caused by the infection of the Gram-negative bacteria Escherichia coli (1). Patients with cystitis often complain of urinary frequency and urgency. Although the pathological basis for these symptoms is still poorly understod, it is thought to be related to the altered bladder microenvironment subsequent to the local influx of inflammatory cells and the production of inflammatory mediators (2, 3).

Urinary frequency and urgency, representing the elevated detrusor excitability, are also typical symptoms of overactive bladder (4). In overactive bladder, these urodynamic dysfunctions are associated with the increased expression and function of gap junctions (GJs)2 in the bladder (5–7). GJs are clusters of transmembrane channels that permit the direct intercellular exchange of ions, secondary messengers, and small signaling molecules. Gap junctional intercellular communication (GJIC) is thought to play an important role in the control of a variety of cellular functions, including cell growth, migration, differentiation, and electric coupling (8, 9). GJs are formed by a family of special proteins termed connexins (Cx). To date, more than 20 different isoforms of Cx molecules have been identified. Among them, connexin 43 (Cx43) has been extensively investigated because of its ubiquitous expression in most cell types. Bladder smooth muscle cells (BSMCs) highly express Cx43 (10). In the normal bladder, Cx43-forming channels provide a pathway for the transmission and propagation of electrical signals, thus contributing to the coordinated contraction and relaxation responses required for normal bladder emptying and filling (5–7). In overactive bladder, Cx43 is abnormally up-regulated and has been shown to be implicated in the enhanced modular autonomous activity and detrusor overactivity (11). In this context, it is likely that the symptoms of bladder infection might also be related to the altered GJs in the bladder. As the first step toward demonstrating this, we examined the changes of GJs during bladder inflammation.

GJs are known to be critically involved in various immune inflammatory situations. On the one hand, they participate in the regulation of almost every step of the inflammatory response, including antigen presentation, chemokine and cytokine production, inflammatory cell migration, adhesion, and

2 The abbreviations used are: GJs, gap junctions; Cx43, connexin43; GJIC, gap junctional intercellular communication; VASP, vasodilator-stimulated phosphoprotein; iNOS, inducible NO synthase; BSMC, bladder smooth muscle cells; L-NAME, L-nitro-arginine methyl ester; RD, rhodamine-dextran; SNAP, S-nitroso-N-acetylpenicillamine; SLDT, scrape-loading dye transfer assay; LY, lucifer yellow; TRITC, tetramethylrhodamine isothiocyanate; CREB, cAMP-response element-binding protein; α-GA, α-glycyrrhetinic acid.
activation (12–17). On the other hand, the proinflammatory mediators LPS, TNFα, and NO have been identified as potent regulators of GJs in a variety of cell types (14, 18–23). There appears to exist an interactive regulation loop between inflammatory and GJs. However, this possibility has not yet been addressed. Another purpose of this study was to test this hypothesis.

Here, we present our evidence showing the existence of an NO-centered regulatory loop between inflammatory cytokines and GJs. Our findings thus provide potentially important insights into the molecular mechanisms of GJs in inflammatory disorders.

**EXPERIMENTAL PROCEDURES**

**Reagents**—IL1β and TNFα were purchased from R&D Systems (Minneapolis, MN). SC514 and anti-iNOS antibodies were obtained from Cayman Chemical Co. Anti-vasodilator-stimulated phosphoprotein (anti-VASP) at serine 157 was obtained from Chemicon International (Temecula, CA). FBS, trypsin/EDTA, antibiotics, LPS, G-nitro-μ-arginine methyl ester (L-NAME), 18 α-glycyrrhetinic acid (α-GA), lindane, heptanol, lucifer yellow (LY), rhodamine-dextran (RD), anti-Cx43, and anti-β-actin antibodies, as well as all other chemicals, were obtained from Sigma.

**Cell Culture**—BSMCs were established from the bladder of female Sprague-Dawley rats as described previously (24). For maintenance, the cells were cultured in DMEM/F-12 containing 10% FBS. For experiments, they were cultured in DMEM/F-12 containing 1% FBS with or without the indicated stimulants. Mouse embryonic fibroblasts were derived from the fetal offspring of mating pairs of heterozygous Cx43 knock-out mice (B6, 129-Gjal<tm 1 Kdr>+/−); The Jackson Laboratory, Bar Harbor, ME), using a method described by Ehlich et al. (25) with minor modifications (26–28). Briefly, both mouse forelimbs were taken from fetuses at day 18 of gestation, minced, and digested in DMEM/F-12 containing 0.1% collagenase for 30 min. Freed cells were collected and cultured in DMEM/F-12 medium containing 15% FBS. Cells at passages 5–15 were used for this study. Genotypes of individual mice were determined by PCR.

**Animals**—Adult female C57BL/6 j mice weighing 25–30 g were purchased from Japan SLC, Inc. (Hamamatsu, Japan). All animal experimental procedures were approved by the Animal Experimental Committee of Yamanashi University. Mice were housed in containment facilities of the Animal Center and maintained on a regular 12:12-h light/dark cycle with food and water.

**Western Blot Analysis**—Western blot was performed using an enhanced chemiluminescence system (22, 23). Briefly, equal amounts of extracted cellular proteins were separated by 10% SDS-polyacrylamide gels and electrotransferred onto polyvinylidene difluoride membranes. After blocking with 3% BSA in PBS, the membranes were incubated with primary 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 (Cell Signaling; Beverly, MA). Immunoreactivity was detected by an enhanced chemiluminescence system (Amersham Biosciences). The chemiluminescent signal was captured with a Fujiﬁlm luminescent image LAS-4000 analyzer (Fujifilm, Tokyo, Japan). To conﬁrm equal loading per lane, membranes were treated with 2% SDS and 100 mm β-mercaptoethanol in 62.5 mm Tris-HCl (pH 6.8) for 30 min at 60 °C and reprobed for β-actin (dilution 1:30,000; Sigma). Data shown are representative of at least three independent experiments with similar results.

**Northern Blot Analysis**—BSMCs were treated with various agents for 12 h. Equal amounts of RNA (5 μg) extracted from cells were separated by electrophoresis and transferred onto nylon membranes (Hybond N+; Amersham Biosciences). The level of Cx43 mRNA was examined as described previously using the entire coding sequence of the rat Cx43 cDNA as a probe (provided by Drs. G. Olbina and W. Eckhart, Molecular and Cell Biology Laboratory, The Salk Institute for Biologic Studies, San Diego). The staining of 28 S and 18 S ribosomal RNA by ethidium bromide was used as a loading control (22, 23).

**Scrape Loading Dye Transfer (SLDT) Assay**—The SLDT assay was used to assess GJIC (27). Cells were exposed to culture medium containing 0.1% LY and 0.05% RD. A scrape line on the monolayer was made with a surgical blade. After washing out background fluorescence, the cells were fixed, and dye transfer results were examined using an Olympus BX50 microscope with a 40× Planapo and FITC (green) or TRITC (red) filter. Immunofluo-
orescence was photographed using a CCD camera attached to the microscope. The distances of LY diffusion from the scrape line were counted for statistical analysis. Because of its large size (10,000 Da), the RD cannot pass through GJs and was used as marker of the scratch-loaded cells. GJIC was evaluated by comparing the diffusion of the LY to the nondiffusible RD.

Measurement of Nitric Levels—NO released into the culture medium was measured by evaluating nitrite accumulation using Griess reagent (29, 30). Briefly, 100 µl of conditioned medium was mixed with an equal amount of Griess reagent (a solution containing 1% sulfanilamide, 0.1% naphthylethylenediamine in 2 M HCl) and allowed to stand at room temperature.
for 10 min. The absorbance at 550 nm was then measured using a microtiter plate reader. Nitrite levels were expressed in nanomoles of NO₂/µg of total cellular protein.

**Statistical Analysis**—Values are expressed as means ± S.D. Comparisons of two populations were made using Student’s t test. For multiple comparisons with a single control, one-way analysis of variance followed by Dunnett’s test was employed. Both analyses were carried out using SigmaStat statistical software (Jandel Scientific). p < 0.05 was considered to be a statistically significant difference.

**RESULTS**

**Proinflammatory Cytokines Increase Cx43 Expression and Function**—To test whether bladder inflammation could affect GJs, we examined Cx43 protein levels after incubation of BSMCs with the major proinflammatory cytokines IL1β and...
Reciprocal Regulation between iNOS and Connexin43

Figure 3. Requirement of NFκB for the cytokine-induced Cx43 expression. A, suppression of the IL1β/TNFα-elicited increase in Cx43 protein levels by an NFκB inhibitor. BSMCs were pretreated with 100 μM SC514 for 30 min before exposing them to 2 ng/ml IL1β and 20 ng/ml TNFα for 24 h. Cellular proteins were extracted. The levels of Cx43 and β-actin were determined by Western blot analysis. B, densitometric analysis of Cx43 expression shown in A. Results are expressed as relative induction compared with the basal level of Cx43 (mean ± S.D., n = 3). #, p < 0.01. C, effect of SC514 on GJIC as evaluated by SLDT assay. The distance of LY diffusion was counted and is expressed as cell layer (mean ± S.D., n = 3). #, p < 0.01.

TNFα. As shown in Fig. 1, A–D, IL1β or TNFα alone caused a moderate increase in Cx43 protein and mRNA levels, while in combination, they exerted a significantly more potent effect. Therefore, the combined stimulation was used for all of the following experiments.

In Western blots, Cx43 protein was detected as three bands (P0, P1, P2; Fig. 1A), corresponding to the nonphosphorylated (P0), phosphorylated (P1), and hyperphosphorylated forms (P2), respectively. IL1β and TNFα induced the native (P0) and phosphorylated forms of Cx43 protein. Time course analysis revealed that the effects of IL1β/TNFα on Cx43 were time-dependent (Fig. 1E). The increased Cx43 levels could be detected 6 h after the stimulation and persisted for at least 48 h.

The elevating effect of IL1β/TNFα on Cx43 expression was confirmed by immunofluorescence staining. Treatment of BSMCs with IL1β/TNFα markedly increased the size and intensity of the punctate Cx43 staining. Normally, Cx43 was mainly localized in the perinuclear region. In the presence of the cytokines, more Cx43 was detected at the region of cell-to-cell contacts (Fig. 1F).

Consistent with the increased localization of Cx43 at the cellular membrane, the GJIC was also increased as evaluated by the SLDT assay. As shown in the Fig. 1, G and H, the distance of LY diffusion (green) from the scrape line in IL1β/TNFα-treated cells was significantly further than that in control cells. Because of the large size, RD (Fig. 1, G and H, red) could not pass through GJs and served as an indicator of the scratch-loaded cells. The merged images are also shown, in which colocalization of LY (Fig. 1, G and H, green) and RD (red) yields yellow. These results indicate that proinflammatory cytokines increase Cx43 expression and promote GJIC in BSMCs.

cAMP Signaling Pathway Mediates IL1β/TNFα-elicited Cx43 Expression—Because cAMP is a well characterized second messenger in the induction of Cx43 and promotion of GJIC (22, 31, 32), one possible mechanism underlying the effect of IL1β/TNFα could be the activation of this pathway. Indeed, exposure of BSMCs to IL1β/TNFα caused a time-dependent increase in the phosphorylation of VASP at serine 157 (Fig. 2A), a validated substrate of cAMP-dependent protein kinase, indicating the activation of PKA (33). In addition, the inhibition of PKA with the PKA inhibitor H89 largely suppressed the cytokine-induced phosphorylation of VASP and Cx43 (Fig. 2, B–D). Consistent with the above finding, H89 also inhibited GJIC, as
revealed by the reduction in LY diffusion (Fig. 2E and supplemental Fig. 1A).

Activated PKA subsequently phosphorylates CREB, leading to the activation of genes that have cAMP-response element-binding sites. Because there are cAMP-response element sites in the Cx43 gene (8, 22), we evaluated their involvement by using CREB siRNA. As shown in Fig. 2, F–H, treatment of BSMCs with the siRNA effectively down-regulated the protein levels of cAMP-response element and concomitantly abolished the increase in Cx43. Therefore, these results indicate that the cAMP signaling pathway mediates the cytokine-induced up-regulation of Cx43.

**NFκB Is Required for IL1β/TNFα-induced Cx43 Expression**—NFκB plays a pivotal role in the cellular inflammatory response (34). Therefore, we examined the possible involvement of NFκB in the Cx43-elevating effect of the cytokines. As shown in Fig. 3, A and B, the inhibition of NFκB with SC514 (50 μM) largely blocked the effect of the cytokines on Cx43 expression (relative levels of Cx43, IL1β/TNFα, 2.69 ± 0.10; IL1β/TNFα + SC514, 1.31 ± 0.14; mean ± S.D.; n = 3). The enhanced GJIC was also suppressed by SC-514 (Fig. 3C and supplemental Fig. 1A). These results indicate that the effect of IL1β/TNFα on Cx43 occurs through the activation of NFκB.

**NO Contributes to the Cytokine-induced Cx43 Elevation**—Among NFκB-regulated gene products, iNOS has been reported to elevate Cx43 expression and function through the cAMP signaling pathway (22, 35). Therefore, we examined the possible involvement of NO. Consistent with the cooperative effects of the cytokines on Cx43, IL1β and TNFα also synergistically elicited iNOS expression (Fig. 4A). In addition, iNOS expression was under the control of NFκB. Inhibition of NFκB with SC-514 completely abrogated iNOS expression (Fig. 4, B and C).

To further establish the role of NO, we examined the effects of interrupting NO generation and action on the expression of Cx43. As shown in Fig. 5, A and B, the iNOS inhibitor l-NAME, NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxy1-3-oxide (cPTIO), and sGC inhibitor oxadiazolo[4,3-a]quinolin1-1-one all significantly blocked the effects of IL1β/TNFα stimulation. C, suppressive effect of l-NAME on IL1β/TNFα-induced GJIC as evaluated by SLDT assay. The distance of LY diffusion was counted and is expressed as cell layer (mean ± S.D., n = 3). #, p < 0.01 versus IL1β/TNFα stimulation. C, suppressive effect of l-NAME on IL1β/TNFα-induced GJIC as evaluated by SLDT assay. The distance of LY diffusion was counted and is expressed as cell layer (mean ± S.D., n = 3). #, p < 0.01. D, induction of Cx43 and pVASP by NO donor SNAP. BSMCs were treated with 200 μM SNAP for the indicated durations. Cellular proteins were extracted. The levels of Cx43 and phosphorylated VASP were determined by Western blot analysis. A β-actin antibody was used as a loading control. E, effect of SNAP on GJIC as evaluated by SLDT assay. BSMCs were treated with 200 μM SNAP for 24 h. The distance of LY diffusion was counted and is expressed as cell layer (mean ± S.D., n = 3), *, p < 0.05 versus untreated control.
Reciprocal Regulation between iNOS and Connexin43

Diphenyleneiodonium, a broad spectrum flavoprotein inhibitor whose targets also include nitric-oxide synthase (37, 38), also significantly inhibited the Cx43-elevating effect. In addition, iNOS inhibitor L-NAME also significantly suppressed the GJIC, as evaluated by SLDT (Fig. 5C and supplemental Fig. 1A). Contrary to the suppressive effects of these inhibitors, the exogenous NO donor SNAP elevated VASP phosphorylation and Cx43 expression in a time-dependent manner, which was associated with an increased GJIC (Fig. 5, D and E, and supplemental Fig. 1B). These results indicate an involvement of NFkB-regulated iNOS in the cytokine-induced up-regulation of Cx43.

Induction of Bladder Cx43 by Intraperitoneal Injection of LPS—To test whether bladder infection and inflammation alter GJs in vivo, we examined the levels of mouse bladder Cx43 after intraperitoneal injection of LPS. As shown in Fig. 6, A and B, LPS induced a time- and dosage-dependent increase in Cx43 protein levels, together with enhanced expression of iNOS. Inhibition of iNOS with L-NAME also significantly blunted the elevation of Cx43 in vivo (Fig. 6, C and D).

Consistent with the elevated Cx43 protein levels, immunofluorescent analysis revealed an increased number of punctate Cx43 spots per field (magnification, ×1200) from four separate experiments (mean ± S.E., n = 4; #, p < 0.01 versus control). For each experiment, an average of five fields were counted.

Inhibition of GJs Potentiates the Cytokine-induced Expression of iNOS in BSMCs—GJs have been implicated in the regulation of various inflammatory responses (12, 15, 17, 20). We therefore asked whether the NO-mediated increase of Cx43 could...
reciprocally affect iNOS expression and NO production. To this end, we examined the change of iNOS and NO after GJ dysfunction caused by three structurally different GJ blockers. As shown in Fig. 7, GJ blockers α-GA, lindane, and heptanol all significantly potentiated the cytokine-triggered expression of iNOS and production of NO. This observation thus suggests that GJs negatively regulate iNOS expression under inflammatory conditions.

**Cx43***/+/* and Cx43**/* Fibroblasts Display Different Responses to IL1β/TNFα-induced iNOS Expression—To further establish the role of Cx43-forming channels in the regulation of iNOS, we have compared the cytokine-induced iNOS expression between fibroblasts derived from Cx43 wild-type (Cx43**/*+) and knock-out (Cx43**/*−/*) littermates. In previous reports, we and others have described that Cx43**/*+/* fibroblasts expressed abundant Cx43 protein and were functionally coupled by GJs, whereas Cx43**/*−/* fibroblasts had neither Cx43 nor functional GJIC (25–28).

First, we confirmed our findings in Cx43**/*+/* fibroblasts. As shown in Fig. 8A, IL1β/TNFα also increased Cx43 expression in fibroblasts. This effect of the cytokines was similarly associated with a parallel increase in the protein levels of iNOS and phosphorylated VASP. In addition, dysfunction of Cx43-forming channels in these cells also influenced the cytokine-elicited expression of iNOS. As shown in Fig. 8, B and C, treatment of Cx43**/*+/* fibroblasts with GJ blockers or specific siRNA amplified the effect of the cytokines on iNOS expression. The effectiveness of Cx43 siRNA in the down-regulation of Cx43 protein levels is shown in Fig. 8C, middle panel. Finally, we compared the iNOS expression and NO production between Cx43**/*+/* and Cx43**/*−/* fibroblasts. Consistent with the above results, IL1β/TNFα induced a significantly higher level of iNOS and NO in Cx43**/*−/* than Cx43**/*+/* fibroblasts (Fig. 8, D–F). Collectively, these results indicate that Cx43-forming channels negatively regulate cytokine-induced iNOS expression.

**DISCUSSION**

In this study, we showed for the first time that the proinflammatory mediators (IL1β, TNFα, and LPS) potently increased Cx43 expression and function in BSMCs. In addition, we established Cx43-forming channels as a negative feedback mechanism in the control of iNOS expression. The major findings and signaling mechanisms involved are schematically depicted in Fig. 9. We have chosen the bladder and cultured BSMCs as a model system for this investigation because the presence of GJs in the bladder and their roles in the regulation of bladder contractility have been well documented. In addition, bladder infection is a common disease. Unraveling the interactive regulation between inflammation and GJs in the bladder could have important clinical and scientific implications.

Exposure of the bladder to LPS markedly elevated Cx43 expression. This effect was similarly achieved by IL1β and TNFα in cultured BSMCs. These observations indicate that bladder infection and inflammation alter GJs in BSMCs. Most of the cellular inflammatory responses are known to be governed by the transcription factor NFκB (34). It was also true for the cytokine-induced expression of Cx43, as shown in this study. Inhibition of NFκB with SC514 completely abolished the increase in Cx43 expression. Several previous papers described the presence of an NFκB-binding site in the promoter region of the Cx43 gene (39, 40). However, the functional role of this site has not been firmly established. In this study, the NFκB-mediated regulation of Cx43 was due to the NFκB-controlled gene products. This notion is supported by the observation that the inhibition of NFκB or the NFκB-regulated gene product iNOS significantly abrogated the Cx43-elevating effects.

The induction of iNOS under inflammatory situations and the implication of NO in the up-regulation of Cx43 have been previously documented (22, 35). Therefore, an involvement of iNOS in elevating Cx43 expression under inflammatory situations is not surprising. The evidence supporting the mediating role of NO also includes that the following. 1) iNOS was under the control of NFκB in BSMCs. 2) The level of iNOS induced by IL1β/TNFα was in parallel to their potency in inducing Cx43 expression. 3) The inhibition of iNOS significantly attenuated the effect of the cytokines. 4) The exog-
Reciprocal Regulation between iNOS and Connexin43

![Diagram of experimental setup](image)

**FIGURE 8. Different response to IL1β/TNFα-induced iNOS expression in Cx43+/+ and Cx43−/− fibroblasts.** A, time-dependent effect of IL1β/TNFα on Cx43, iNOS, and pVASP expression in Cx43+/+ fibroblasts. Cx43−/− fibroblasts were exposed to 2 ng/ml IL1β and 20 ng/ml TNFα for the indicated duration. Cellular proteins were subjected to Western blot analysis for iNOS, pVASP, and Cx43. β-Actin was used as a loading control. B, effects of various GJ blockers on iNOS expression. Cx43−/− fibroblasts were pretreated with 20 μM α-GA, 100 μM indane, and 4 mM heptanol for 30 min and exposed to 2 ng/ml IL1β and 20 ng/ml TNFα for an additional 24 h. The cellular proteins were extracted and assayed for iNOS and β-actin levels by Western blot analysis. C, effect of Cx43 siRNA on iNOS expression. Cx43−/− fibroblasts were transfected with control or Cx43 siRNA for 24 h. The cells were then exposed to IL1β/TNFα for an additional 24 h. Cellular proteins were analyzed for iNOS, Cx43, and β-actin. D, different induction of iNOS by IL1β/TNFα in Cx43+/+ and Cx43−/− fibroblasts. Cx43+/+ and Cx43−/− fibroblasts were exposed to 2 ng/ml IL1β and 20 ng/ml TNFα for the indicated duration. The cellular proteins were assayed for iNOS, Cx43, and β-actin levels by Western blot analysis. E, densitometric analysis of iNOS expression shown in D. Results were expressed as levels relative to the zero point control (mean ± S.D., n = 3), #, p < 0.01. F, induction of NO release by IL1β/TNFα in Cx43+/+ and Cx43−/− fibroblasts. Cx43+/+ and Cx43−/− fibroblasts were exposed to 2 ng/ml IL1β and 20 ng/ml TNFα for the indicated duration. The conditioned media were harvested and assayed for nitrite concentration. Results are expressed as level relative to the basal NO2 concentration in Cx43−/− cells (mean ± S.D., n = 4), #, p < 0.01.

Enous NO donor SNAP similarly induced the phosphorylation of VASP and Cx43.

In addition to BSMCs, IL1β/TNFα also elevated Cx43 protein levels in glomerular mesangial cells, renal tubular epithelial cells (data not shown), and fetal forearm fibroblasts. In addition, Cx43 in the bladder epithelial cells was also induced by intraperitoneal injection of LPS. Interestingly, the combined stimulation of DCs and macrophages with LPS, TNFα, and IFN-γ also increased Cx43 expression in these cells (12, 41). It is possible that similar regulatory mechanisms operate in these cells. Of note, in contrast to our results, there are also reports documenting a suppressive effect of proinflammatory mediators (LPS, TNFα, and NO) on GJs (42, 43). The reasons for this discrepancy are presently unclear. It could be due to the different cell types and experimental settings used for the investigation. More detailed analysis to account for the difference may be needed in the future.

The increased Cx43 expression by NO could contribute to the altered sensitivity of bladder detrusor to stimuli. In a recent report, *E. coli*-induced bladder inflammation was associated with an increased bladder contractile response, which was mediated by the NO-induced activation of PKC (44). Given that GJs are required for the coordinated contractile response (45), the elevation of Cx43 by NO in BSMCs could exaggerate the abnormal bladder contractile response under inflammatory situations.

There is growing evidence indicating a critical involvement of GJs in immune inflammatory processes. Depending on the cell types, isoforms of Cxs and employed stimuli, GJs could either promote or suppress the inflammatory responses (20, 46, 47). For example, in DC cells, we have demonstrated that GJs contribute to their activation and production of inflammatory mediators (14). In contrast, Cx32 in endothelial cells has been recently documented to suppress TNFα-induced production of
Reciprocal Regulation between iNOS and Connexin43

FIGURE 9. Schematic diagram illustrating reciprocal regulation between iNOS and Cx43 under inflammatory situations. Inflammatory stimuli elicit an NFkB-dependent expression of iNOS and production of NO, which activates the cAMP signaling pathway, leading to increased Cx43 expression and function. Meanwhile, the increased Cx43 exerts negative feedback regulation on iNOS expression and NO production.

IL-6 and MCP-1 (48). Here, we established Cx43-forming channels as a negative feedback mechanism for cytokine-induced iNOS expression. Currently, the molecules and signaling mechanisms involved in the regulatory effects of GJs on iNOS are still unclear. GJs are known to regulate cell functions through GJIC-dependent or -independent actions. The suppressive effect of GJs on iNOS could be related to the increased intercellular communication because the GJ inhibitor heptanol that disrupts GJIC by decreasing membrane fluidity without affecting Cx43 (49, 50) also significantly potentiated the cytokine-induced iNOS expression. The molecules transmitted by GJs remain to be characterized. The NO-centered regulatory loop between inflammation and GJs, as revealed in this study, might provide a molecular link between intercellular and paracrine pathways under inflammatory situations, which may have an important impact on the initiation and development of inflammatory disorders.

Collectively, we established a reciprocal regulation loop between iNOS and GJs in BSMCs. Our findings thus provide a potentially important molecular mechanism for urgency symptoms of bladder infection. In addition, it may open a new window toward our understanding of the role of GJs in the pathogenesis of inflammatory disorders.

REFERENCES

1. Foxman, B., and Brown, P. (2003) Infect. Dis. Clin. North Am. 17, 227–241.
2. Bills, B. K., Forrestal, S. G., Ryczek, M. T., Johnson, J. R., Klumpp, D. J., and Schaeffer, A. J. (2007) Infect. Immun. 75, 5353–5360.
3. Bouchelouche, K., Alvarez, S., Horn, T., Nordling, J., and Bouchelouche, P. (2006) Urology 67, 214–219.
4. Wein, A. J., and Rosneck, E. S. (2002) Urology 60, 7–12.
5. Christ, G. J., Day, N. S., Day, M., Zhao, W., Persson, K., Pandita, R. K., and Andersson, K. E. (2003) Am. J. Physiol. Regul. Integr. Comp. Physiol. 284, R1241–R1248.
6. Haferkamp, A., Mundhenk, J., Bastian, P. J., Reitz, A., Dörsam, J., Pannek, J., Schumacher, S., Schurch, B., Böttner, R., and Müller, S. C. (2004) Eur. Urol. 46, 799–805.
7. Li, L., Jiang, C., Hao, P., Li, W., Song, C., and Song, B. (2007) Am. J. Physiol. Cell Physiol. 293, C1627–C1635.
8. Saez, J. C., Berthoud, V. M., Branes, M. C., Martinez, A. D., and Beyer, E. C. (2003) Physiol. Rev. 83, 1359–1400.
9. Yao, J., Oite, T., and Kitamura, M. (2009) J. Am. Physiol. Renal Physiol. 296, F939–F946.
10. Neuhaus, J., Weimann, A., Stolzenburg, J. U., Wolburg, H., Horn, L. C., and Dorschner, W. (2002) World J. Urol. 20, 250–254.
11. Drake, M. J., Hedlund, P., Harvey, I. J., Pandita, R. K., Andersson, K. E., and Gillespie, J. I. (2003) J. Urol. 170, 276–279.
12. Eugenin, E. A., Brañes, M. C., Berman, J. W., and Sáez, J. C. (2003) J. Immunol. 170, 1320–1328.
13. Kasper, C. A., Sorg, I., Schnitz, C., Tschon, T., Wischnewski, H., Kim, M. L., and Arriemouloou, C. (2010) Immunity 33, 804–816.
14. Matsue, H., Yao, J., Matsue, K., Nasagasa, A., Sugiyama, H., Aoki, R., Kitamura, M., and Shimada, S. (2006) J. Immunol. 176, 181–190.
15. Neijissen, J., Herberts, C., Drijfhout, J. W., Reits, E., Janssen, L., and Neefjes, J. (2005) Nature 434, 83–88.
16. Stuehr, D. J., Fasehun, O. A., Kwon, N. S., Gross, S. S., Gonzalez, J. A., Levi, R., and Nathan, C. F. (1991) J. Immunol. 147, 491–497.
17. Véliz, E. A., González, H. E., Sánchez, H. A., Brañes, M. C., and Sáez, J. C. (2003) Cell. Immunol. 247, 103–110.
18. Neuhaus, J., Heinrich, M., Schwalenberg, T., and Stolzenburg, J. U. (2009) Eur. Urol. 55, 491–497.
19. Parthasarathi, K., Ichimura, H., Monna, E., Lennert, J., Quadri, S., Issekkutz, A., and Bhattacharya, J. (2006) J. Clin. Invest. 116, 2193–2200.
20. Scheckenbach, K. E., Crespin, S., Kwak, B. R., and Chanson, M. (2011) J. Vasc. Res. 48, 91–102.
21. Yao, J., Hiramatsu, N., Zhu, Y., Morioka, T., Takeda, M., Oite, T., and Kitamura, M. (2005) J. Am. Soc. Nephrol. 16, 58–67.
22. Yao, J., Kitamura, M., Zhu, Y., Meng, Y., Kasai, A., Hiramatsu, N., Morioka, T., Takeda, M., and Oite, T. (2006) J. Am. Physiol. Renal Physiol. 290, F1083–F1093.
23. Sawada, N., Yao, J., Hiramatsu, N., Hayakawa, K., Araki, I., Takeda, M., and Kitamura, M. (2008) Lab. Invest. 88, 553–563.
24. Ehrlich, H. P., Gabbiani, G., and Meda, P. (2000) J. Cell. Physiol. 184, 7–21.
25. Fang, X., Huang, T., Zhu, Y., Yan, Q., Chi, Y., Jiang, J. X., Wang, P., Matsue, H., Kitamura, M., and Yao, J. (2011) Antioxid. Redox. Signal. 14, 2427–2439.
26. Huang, T., Zhu, Y., Fang, X., Chi, Y., Kitamura, M., and Yao, J. (2010) Cancer Sci. 101, 713–721.
27. Yao, J., Huang, T., Fang, X., Chi, Y., Zhu, Y., Wan, Y., Matsue, H., and Kitamura, M. (2010) Br. J. Pharmacol. 160, 2055–2068.
28. Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., and Tannenbaum, S. R. (1982) Anal. Biochem. 126, 131–138.
29. Yao, J., Zhu, Y., Sun, W., Sawada, N., Hiramatsu, N., Takeda, M., and Kitamura, M. (2007) Br. J. Pharmacol. 151, 457–466.
30. Dowling-Warriner, C. V., and Tosko, E. J. (2000) Neuroscience 95, 859–868.
31. Paulson, A. F., Lampe, P. D., Meyer, R. A., TenBroek, E., Atkinson, M. M., and Dorschner, W. (2002) World J. Urol. 20, 1320–1328.
32. Alonso, F., Krattinger, N., Mazzolai, L., Simon, A., Waeber, G., Meda, P., and Steinberg, T. H. (1998) J. Cell. Biochem. 70, 8–21.
33. Imenez-Felstrom, J., Lundquist, I., and Salehi, A. (2005) Cell Tissue Res. 319, 221–230.
34. Stuehr, D. J., Fasehun, O. A., Kwon, N. S., Gross, S. S., Gonzalez, J. A., Levi, R., and Nathan, C. F. (1991) FASEB J. 5, 98–103.
35. Wu, F., Tyml, K., and Wilson, J. X. (2008) J. Cell. Physiol. 217, 207–214.
36. Alonso, F., Krattinger, N., Mazzolai, L., Simon, A., Waeber, G., Meda, P., and Steinberg, T. H. (2010) Cardiovasc. Res. 87, 166–176.
37. Zhao, Y., Rivieccio, M. A., Lutz, S., Scemes, E., and Brosnan, C. F. (2006) Glia 54, 775–785.
38. Fernandez-Cobo, M., Gingalewski, C., and De Maio, A. (1998) Shock 10, 97–102.
Reciprocal Regulation between iNOS and Connexin43

42. Liao, C. K., Wang, S. M., Chen, Y. L., Wang, H. S., and Wu, J. C. (2010) Int. J. Biochem. Cell Biol. 42, 762–770
43. Simon, A. M., McWhorter, A. R., Chen, H., Jackson, C. L., and Ouellette, Y. (2004) J. Vasc. Res. 41, 323–333
44. Weng, T. I., Wu, H. Y., Lin, P. Y., and Liu, S. H. (2009) Infect. Immun. 77, 3312–3319
45. Yao, J., Morioka, T., Li, B., and Oite, T. (2002) J. Am. Soc. Nephrol. 13, 2018–2026
46. Kielian, T. (2008) J. Neurochem. 106, 1000–1016
47. Mori, R., Power, K. T., Wang, C. M., Martin, P., and Becker, D. L. (2006) J. Cell Sci. 119, 5193–5203
48. Okamoto, T., Akiyama, M., Takeda, M., Akita, N., Yoshida, K., Hayashi, T., and Suzuki, K. (2011) Exp. Cell Res. 317, 348–355
49. Cronier, L., Frendo, J. L., Defamie, N., Pidoux, G., Bertin, G., Guibourdenche, J., Pointis, G., and Malassine, A. (2003) Biol. Reprod. 69, 1472–1480
50. Takens-Kwak, B. R., Jongsma, H. J., Rook, M. B., and Van Ginneken, A. C. (1992) Am. J. Physiol. 262, C1531–C1538