Phenotypic modulation of cultured bladder smooth muscle cells and the expression of inducible nitric oxide synthase

Rebecka Johansson1 and Katarina Persson1,2
1Department of Clinical and Experimental Pharmacology, Lund University Hospital, SE-221 85 Lund and
2Department of Chemistry and Biomedical Sciences, University of Kalmar, SE-391 82 Kalmar, Sweden

Submitted 5 August 2003; accepted in final form 28 November 2003

Johansson, Rebecka, and Katarina Persson. Phenotypic modulation of cultured bladder smooth muscle cells and the expression of inducible nitric oxide synthase. Am J Physiol Regul Integr Comp Physiol 286: R642–R648, 2004. First published December 4, 2003; 10.1152/ajpregu.00443.2003.—Phenotypic modulation of smooth muscle is associated with various pathological conditions, including bladder dysfunction. Cytoskeletal dynamics modulate the cell phenotype and were recently shown to be involved in regulation of inducible nitric oxide synthase (iNOS). We tested the hypothesis that the cell differentiation status affects iNOS expression, and that iNOS is preferentially expressed in immature dedifferentiated bladder smooth muscle cells (BSMC). Isolated rat BSMC were put into different stages of differentiation by serum deprivation on laminin-coated plates in the presence of IGF-I and by interaction with Rho signaling and actin polymerization. iNOS and smooth muscle-myosin heavy chain (SM-MHC) protein expression were investigated with Western blot analysis. Our results showed iNOS protein in BSMC exposed to interleukin-1β (2 ng/ml) + TNF-α (50 ng/ml). Growth of BSMC in serum-free medium on laminin in the presence of IGF-I increased SM-MHC expression, whereas cytokine-induced iNOS was inhibited. Disruption of F-actin with latrunculin B (0.5 μM) potentiated iNOS expression and decreased SM-MHC expression. Rho inhibition with C3 (2.5 μg/ml) increased iNOS expression, whereas SM-MHC expression was slightly decreased. Rho-kinase inhibition with Y-27632 (10 μM) mediated a decrease in iNOS and a slight increase in SM-MHC expression. In conclusion, the capacity of BSMC to express iNOS was negatively correlated to differentiation status measured as SM-MHC expression. Actin cytoskeletal dynamics and Rho signaling are involved in regulation of cytokine-induced iNOS expression in BSMC. Phenotypic changes and impairment in actin cytoskeleton formation may potentiate cytokine activation and in turn increase nitric oxide production in the bladder during disease differentiation; F-actin; nitric oxide; Rho; bladder; smooth muscle-myosin heavy chain

PHENOTYPIC MODULATION of smooth muscle is associated with various pathological conditions, including atherosclerosis (35), asthma (13), and bladder dysfunction (6). In progress of disease, the smooth muscle may change phenotype from differentiated to dedifferentiated (11, 33). Bladder outlet obstruction is associated with phenotypic remodeling with alterations in the cytoskeletal and contractile protein composition (5, 6). During interstitial cystitis, bladder smooth muscle cells (BSMC) are observed to express a more immature and synthetic smooth muscle phenotype (34).

The extracellular matrix regulates phenotype and proliferation (14, 30) and during inflammation and injury the extracellular matrix may be altered and mediate cytoskeletal changes. Actin cytoskeletal dynamics regulate smooth muscle-specific gene expression and thus modulate changes in the cell phenotype (21, 28, 46).

Multiple signaling pathways regulate smooth muscle cell differentiation, and recently much attention has been focused on Rho signaling (11, 21). Rho proteins are regulators of the actin cytoskeleton, gene transcription, cell cycle progression, and adhesion (22). The effects of Rho on smooth muscle differentiation may be secondary to the action of Rho on the actin cytoskeleton (21, 39). The transcription factor serum response factor (SRF) is involved in smooth muscle differentiation (24). The actin cytoskeleton is shown to regulate SRF (39), which in turn regulates the expression of smooth muscle cell-specific genes.

Inducible nitric oxide synthase (iNOS) is induced in response to proinflammatory stimuli. BSMC do not express iNOS under normal conditions, but expression may be induced upon stimulation with proinflammatory cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) (17, 44). The IL-1 signaling pathway involves Rho and interactions with actin polymerization (37). Interestingly, the cytoskeleton has been shown to affect iNOS expression (43, 47). Disruption of actin polymerization increased iNOS expression in glomerular mesangial and epithelial cells (43, 47). Thus cytoskeletal dynamics may not only affect the differentiation status of BSMC but also regulate iNOS expression.

In a previous study, we demonstrated that approximately one-third of cytokine-stimulated BSMC in culture expressed iNOS (17). We speculated that the iNOS-expressing cells had a common phenotype or state of differentiation. In this study, we tested the hypothesis that iNOS is preferentially expressed in immature dedifferentiated BSMC. Isolated BSMC were put into different stages of differentiation by serum deprivation on laminin-coated plates and by interactions with Rho signaling and actin polymerization.

MATERIALS AND METHODS

Cell culture. Primary cell cultures were prepared from bladders of female Sprague Dawley rats (200 g). The rats were killed by CO2 asphyxia. The experimental protocol was approved by the Animal Ethics Committee (Lund University, Lund, Sweden). The bladder was carefully cleaned from fat and connective tissue and scraped with a scalpel to remove the mucosa and serosa. Four bladders were minced in sterile PBS and incubated with sterile trypsin (10 mg/ml; Sigma, St. Louis, MO) and collagenase XI (2 mg/ml; Sigma) dissolved in PBS for 45–60 min at 37°C. The dissociated cells were plated on tissue culture dishes in DMEM-F-12 (Sigma) supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Address for reprint requests and other correspondence: R. Johansson, Dept. of Clinical and Experimental Pharmacology, Lund Univ. Hospital, SE-221 85 Lund, Sweden (E-mail: Rebecka.Johansson@klinfarm.lu.se).

0363-6119/04 $5.00 Copyright © 2004 the American Physiological Society http://www.ajpregu.org
(all from Sigma). Cells were incubated at 37°C in humidified air with 5% CO₂. The cells were continuously subcultured when confluent. All studies were performed on cells in passages 3–5 and from at least two different primary cell cultures.

BSMC were stimulated with the mouse recombinant cytokines IL-1β (2 ng/ml; Sigma) and TNF-α (50 ng/ml; Sigma) for the last 24 h in each experiment. These cytokines are known as effective inducers of iNOS in cell lines. BSMC were incubated with Y-27632 (10 μM; Tocris, Bristol, UK), which is a Rho-kinase inhibitor (27), for 48 h and with latrunculin B (LatB; 0.5 μM; Calbiochem, La Jolla, CA), which disrupts F-actin formation (40), for 1–24 h.

In some experiments, BSMC were stimulated with the nitric oxide (NO) donor DETA-NONOate (100–300 μM; Alexis Biochemicals, Lausen, Switzerland) for 48 h and the NO and superoxide donor 3-morpholinosydnonimine (SIN-1) (100–500 μM; Calbiochem) for 24 h.

Laminin-cultured BSMC. BSMC were cultured on laminin-coated tissue culture plates (BD Biosciences, Bedford, MA) in serum-free medium with additional supplement of 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml sodium selenite (all from Sigma). BSMC were cultured in the presence of insulin-like growth factor-1 (IGF-1; 20 ng/ml; Sigma) for 72 h and with or without IL-1β + TNF-α during the last 24 h. In some experiments, BSMC were stimulated with platelet-derived growth factor-BB (PDGF; 10 ng/ml; Sigma) for 24 h and then cultured an additional 24 h with or without cytokines in the absence of PDGF.

Permeabilization with lipofectamine and C3 stimulation. Introduction of C3 exoenzyme into BSMC to inhibit Rho (1) was done according to a method by Bobiriev et al. (4). BSMC grown confluent on 60-cm² petri dishes were incubated with lipofectamine (5 μg/ml; Invitrogen, Life Technologies, Carlsbad, CA) for 45 min. C3 exoenzyme (2.5 μg/ml; List Biological Laboratories, Campbell, CA) was added, and the cells were incubated for 10 h. The cells were washed in PBS and incubated with IL-1β + TNF-α for 24 h.

F-actin staining and immunohistochemical detection of iNOS. BSMC were cultured in four-well chamber slides (Nalge Nunc International, Naperville, IL) and incubated with LatB, Y-27632, and C3 (see above). The cells were washed with PBS and fixed with 4% formaldehyde in PBS for 15 min. Thereafter, the cells were treated with 0.05% Triton X-100 (Sigma) and 0.2% BSA in PBS for 30 min at 37°C. Cells were stained for F-actin with Texas Red-X phalloidin (Molecular Probes, Eugene, OR) for 20 min.

In some experiments, BSMC were stimulated with IL-1β + TNF-α for 24 h and fixed as described above. Immunohistochemistry was performed with a rabbit polyclonal antibody raised against iNOS (1:1,000; Santa Cruz Biotechnology). As secondary antibody, FITC-conjugated F(ab’)_2 fragment of goat anti-rabbit IgG (1:80; Jackson ImmunoResearch Laboratories, West Grove, PA) was used. Thereafter, F-actin staining with Texas Red-X phalloidin was performed as described above. All dilutions were made in 0.05% Triton X-100 and 0.2% BSA in PBS.

The slides were finally rinsed in PBS and mounted in PBS/glycerol with p-phenylenediamine to prevent fading. All micrographs were obtained using a digital camera system (Nikon E400 microscope and Optronix DEI-750 camera), and the pictures were captured using appropriate filter settings for FITC and Texas Red. Adobe Photoshop was used for image handling, and the color channels were handled separately. Only the background, contrast, and brightness of the entire image were changed in the final picture.

Western blot analysis. BSMC cultured in 60-cm² dishes were analyzed by Western blot for detection of iNOS protein expression and smooth muscle-myosin heavy chain (SM-MHC). The cells were washed in sterile PBS and lysed in buffer containing 10% glycerol, 2% β-mercaptoethanol, 10 mM Tris-HCl (pH 7.4). The protein content was determined by the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA) with BSA as standard (Pierce, Rockford, IL). β-Mercaptoethanol (5%; Sigma) and bromphenol blue were added, and the samples were boiled for 5 min. Samples containing 10–25 μg protein were separated by 7.5% SDS-PAGE (Bio-Rad Laboratories) and blotted onto a polyvinylidine difluoride membrane (Bio-Rad Laboratories). The membrane was blocked with 5% nonfat milk overnight at 4°C and incubated with a rabbit polyclonal antibody raised against iNOS (1:1,000; Santa Cruz Biotechnology) or a mouse monoclonal antibody raised against SM-MHC (1:500; Santa Cruz Biotechnology) for 1 h. Subsequently, the membrane was incubated with a secondary donkey anti-rabbit IgG antibody conjugated with horseradish peroxidase (1:10,000; Santa Cruz Biotechnology) or an anti-mouse IgG antibody with horseradish peroxidase (1:10,000; Santa Cruz Biotechnology) for 1 h in room temperature. The bound antibodies were visualized using enhanced chemiluminescence reagents and exposed to X-ray film (both from Amersham Pharmacia Biotech, Buckinghamshire, UK). Densitometry was performed on the Western blots using Bio-Rad Gel Doc 2000 and the data analysis program Bio-Rad Quantity One. Results are expressed as the mean percent of control.

RESULTS

Laminin and IGF-I inhibit iNOS expression. BSMC were cultured on laminin-coated plates in serum-free medium supplemented with IGF-I (20 ng/ml). This combination is known to induce a differentiated smooth muscle phenotype (12). SM-MHC is suggested to be a marker for differentiated smooth muscle cells (25, 38) and was used to study the differentiation state of the BSMC.

BSMC cultured without laminin and in the presence of serum showed no or weak SM-MHC expression (Fig. 1A). Cells cultured on laminin-coated plates in serum-free medium supplemented with IGF-I (20 ng/ml) demonstrated increased SM-MHC expression, as detected by Western blot analysis (Fig. 1A and Table 1). BSMC grown on laminin and in the presence of PDGF (10 ng/ml), a known mitogen that dedifferentiates smooth muscle cells, showed a weaker SM-MHC expression compared with IGF-I-stimulated cells (Fig. 1A).

iNOS protein expression was detected in IL-1β + TNF-α-stimulated BSMC cultured without laminin and in the presence of serum as well as in cytokine-stimulated cells cultured on laminin in the presence of PDGF (Fig. 1B). However, IL-1β + TNF-α only induced a weak iNOS expression in IGF-I-treated BSMC grown on laminin (Fig. 1B and Table 1). IGF-I per se did not reduce cytokine-induced iNOS expression in BSMC.
grown without laminin and in the presence of serum (data not shown).

Disruption of actin polymerization stimulates iNOS expression. BSMC stimulated with IL-1β + TNF-α for 24 h were stained for iNOS (FITC) and for F-actin with Texas Red-X phalloidin. BSMC that expressed iNOS had low F-actin expression (Fig. 2, A–C).

Incubation with LatB (0.5 μM) for 1 h impaired F-actin formation, and the filaments were reorganized, as confirmed by immunohistochemistry (Fig. 2, D and E). SM-MHC expression was reduced after LatB treatment for 24 h, as detected with Western blot analysis (Fig. 3A and Table 1). IL-1β + TNF-α induced iNOS protein expression in BSMC after 24 h, and the expression was increased in the presence of LatB (Fig. 3B and Table 1). LatB alone did not induce iNOS (data not shown). Stimulation with IL-1β alone induced iNOS, and LatB further increased this expression (Fig. 3C). However, TNF-α did not induce iNOS protein, alone or in combination with LatB (Fig. 3C). Time course studies showed that IL-1β induced iNOS protein expression after 6 h and that the expression increased

Table 1. Quantitative analysis of IL-1β + TNF-α-induced iNOS expression and SM-MHC expression by densitometry of Western blots

<table>
<thead>
<tr>
<th></th>
<th>iNOS, % IL-1β + TNF-α-Stimulated Cells</th>
<th>SM-MHC, % Control Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>LatB</td>
<td>191</td>
<td>64</td>
</tr>
<tr>
<td>C3</td>
<td>363</td>
<td>62</td>
</tr>
<tr>
<td>Y-27632</td>
<td>73</td>
<td>176</td>
</tr>
<tr>
<td>IGF-I</td>
<td>18</td>
<td>346</td>
</tr>
</tbody>
</table>

SM-MHC, smooth muscle-myosin heavy chain; LatB, latrunculin B; n = 2–5 experiments.

Fig. 3. Effect of LatB on SM-MHC and iNOS protein expression in BSMC as detected with Western blot analysis. SM-MHC expression in BSMC stimulated with LatB for 24 h (A) and iNOS expression in BSMC stimulated with IL-1β + TNF-α alone and in combination with LatB for 24 h (B). C: effect of the individual cytokines IL-1β and TNF-α alone and in combination with LatB for 24 h. D: effect of LatB on IL-1β-induced iNOS protein expression at various times. Representative results from 2–3 separate experiments.
over time (Fig. 3D). LatB potentiated the IL-1β-induced iNOS expression at all times. In the presence of LatB, IL-1β induced iNOS as early as 3 h after stimulation.

**Interactions with Rho signaling affect iNOS expression.** Incubation with the Rho-kinase inhibitor Y-27632 (10 μM) for 48 h increased the SM-MHC expression, as observed by Western blot analysis (Fig. 4A and Table 1). iNOS protein expression induced by IL-1β + TNF-α decreased by incubation with Y-27632 (Fig. 4B and Table 1). Y-27632 alone did not induce iNOS protein expression (data not shown). Inhibition of Rho with C3 for 34 h decreased SM-MHC expression, as detected with Western blot analysis (Fig. 4C and Table 1). C3 treatment increased the cytokine-induced iNOS expression (Fig. 4D and Table 1). C3 alone did not induce iNOS (data not shown). The F-actin organization was not affected by Y-27632 or C3 uptake when investigated by Texas Red-X phalloidin (data not shown).

**NO increases SM-MHC expression.** BSMC incubated with the NO donor DETA-NONOate (100–300 μM) for 48 h and the NO and superoxide donor SIN-1 (100–500 μM) for 24 h increased the expression of SM-MHC, as detected with Western blot analysis (Fig. 5). DETA-NONOate at 100 μM and SIN-1 at 100 μM increased SM-MHC expression to 122 and 180%, respectively, compared with control.

**DISCUSSION**

Smooth muscle cells in culture do not maintain their differentiated phenotype but converge to a dedifferentiated state (8). Smooth muscle cells cultured on laminin-coated plates in serum-free medium with IGF-I are shown to maintain a differentiated phenotype but converge to a dedifferentiated state (8). Passaged cultured smooth muscle cells can, however, never become completely differentiated, a phenomenon has, however, been investigated in correlation to iNOS expression in the vasculature. The intimal smooth muscle cells exhibit a different morphological phenotype from smooth muscle cells in the media and show a higher activity of the iNOS promoter in response to inflammatory stimuli (45). The finding that differentiation status is coupled to iNOS expression should be taken into account when designing experiments for investigation of iNOS expression. Experimental iNOS induction may be favored by using cells in a dedifferentiated state.

We next investigated cytoskeletal dynamics in relation to iNOS induction. Disruption of the actin filament with LatB potently increased cytokine-induced iNOS expression in BSMC in line with findings in glomerular mesangial and epithelial cells (43, 47). Impairment in the actin cytoskeleton formation may thus potentiate cytokine activation and in turn increase NO production in the bladder during disease. The mechanism for the increased iNOS response after LatB was not investigated in our study, but other studies have shown that LatB inhibits signal transduction to SRF by blocking actin polymerization (39). SRF has been shown to contribute to the regulation of smooth muscle differentiation marker genes and may thus be a regulator of smooth muscle cell differentiation (7). Indeed, a decrease in SM-MHC expression was observed in LatB-treated BSMC cells. The stimulatory effect on iNOS expression in dedifferentiated BSMC and in LatB-treated cells may be the result of a lower influence of SRF in these cells (see Fig. 6). SRF is shown to exert a negative regulatory role on iNOS induction in glomerular mesangial cells (47).

Rho is involved in the regulation of smooth muscle differentiation marker genes and in actin filament organization (21; Fig. 6). It is suggested that Rho-dependent changes in actin polymerization modulate gene transcription by regulating the activity of SRF (21, 39). Treatment of BSMC cells with the Rho inhibitor C3 decreased SM-MHC expression and increased iNOS expression in cytokine-stimulated BSMC. Thus, again, a negative correlation between iNOS and SM-MHC expression could be demonstrated. Several other studies have demonstrated that inhibition of the Rho pathway with C3 enhances the expression of iNOS protein (10, 26), suggesting an inhibitory role for Rho in iNOS. Our data suggest that the increased iNOS expression caused by growth factor PDGF, which dedifferentiates smooth muscle, decreased SM-MHC expression in BSMC grown on laminin.

We found a negative correlation between the expression of SM-MHC and cytokine-induced iNOS protein expression, indicating that the expression of iNOS may be related to the differentiation status of smooth muscle cells. High SM-MHC expression was coupled to low iNOS expression, indicating that differentiated smooth muscle cells are less capable of expressing iNOS. This correlation has to our knowledge not been previously observed in BSMC. The smooth muscle phenotype has, however, been investigated in correlation to iNOS expression in the vasculature. The intimal smooth muscle cells exhibit a different morphological phenotype from smooth muscle cells in the media and show a higher activity of the iNOS promoter in response to inflammatory stimuli (45). The finding that differentiation status is coupled to iNOS expression should be taken into account when designing experiments for investigation of iNOS expression. Experimental iNOS induction may be favored by using cells in a dedifferentiated state.

![Fig. 4. Effect of Rho and Rho-kinase inhibition on iNOS and SM-MHC expression as detected by Western blot analysis. A: SM-MHC expression in BSMC incubated with Y-27632 (10 μM) for 48 h. B: iNOS expression in Y-27632 and IL-1β + TNF-α (24 h)-treated cells. C: SM-MHC expression in C3-treated BSMC. D: iNOS protein expression in IL-1β + TNF-α-stimulated C3-treated cells. Representative results from 3 separate experiments.](image-url)
inhibition of Rho may be associated with effects on cell differentiation.

The effect of Rho on actin fibers involves downstream targets such as Rho-kinase, which can be inhibited by compound Y-27632 (23). In the urinary bladder, Rho-kinase is reported to be involved in smooth muscle contraction (42), whereas a role for Rho-kinase in phenotypic modulation and iNOS induction is to our knowledge not known. To our surprise, the Rho-kinase inhibitor Y-27632 slightly increased SM-MHC expression, whereas the cytoskeleton-mediated iNOS expression was decreased. Kraynack et al. (19) reported that inhibition of Rho-kinase with Y-27632 decreased iNOS promoter activity although the iNOS mRNA and protein was increased. The involvement of Rho and Rho-kinase in iNOS induction appears to be complex. The divergent effects of Rho inhibition with C3 and Rho-kinase inhibition with Y-27632 on iNOS induction may be the result of different levels of inhibition of the Rho pathway (Fig. 6). Rho mediates actin and stress fiber formation by different pathways. Besides Rho-kinase, Rho regulates the downstream proteins Dia/profilin that are also required for actin and stress fiber polymerization (11, 41). Thus, after inhibition of Rho-kinase, actin and stress fiber polymerization may still occur through the Dia/profilin pathway (Fig. 6). This may explain the opposing effects on iNOS and SM-MHC expression found in BSMC when inhibiting Rho and Rho-kinase.

The effects on iNOS after interference with Rho signaling may in part be independent of actin polymerization and cell differentiation. We did, e.g., not observe any effect of C3 or Y-27632 on F-actin organization when investigated with immunohistochemistry (data not shown). Recent studies show that Rho is a negative regulator of NF-κB (32), a well-known activator of the iNOS promoter, whereas Rho-kinase is observed to activate NF-κB (36). This may explain the divergent effects of C3 and Y-27632 on iNOS expression (Fig. 6). Moreover, Rho is shown to enhance the activity of the transcription factor activating protein-1 (AP-1; see Ref. 9), which is a negative regulator of iNOS induction (18). AP-1 was recently suggested to be involved in Rho-dependent inhibition of cytokine-induced iNOS expression (43).

The iNOS gene is normally regulated at various levels of synthesis and degradation (31). Once induced, iNOS produces sustained amounts of NO that may exert tissue-damaging effects when produced in high concentrations by forming the cytotoxic peroxynitrite (3). The cytotoxic effects of NO are nonspecific, and the consequences of overproduction of NO can be detrimental to the host. Therefore, a precise regulation of NO production would be critical for the normal function of the host defense system as well as for the survival of the host cells. Our results suggest that the differentiation status of the cell may be considered as a regulator of iNOS expression. During injury and inflammation, actin polymerization and cell differentiation may be affected, and the negative regulation on iNOS induction may be withdrawn. Phenotypic modulation and reorganization of the actin cytoskeleton after pathological conditions may be a means to facilitate iNOS and modulate the inflammatory response.

We observed an increase in SM-MHC protein expression in BSMC exposed to exogenous NO, in line with earlier findings in other cell types (15, 20). NO is produced in the bladder under normal conditions from nerves expressing neuronal NOS (29). However, NO has only minor effects on bladder smooth muscle tone (2), and the functional significance of NO in the bladder has been unclear. One role of NO in the bladder may be, as indicated from the results in our study, to stabilize and maintain the differentiated phenotype of the smooth muscle cells. During bladder outlet obstruction, accompanied with smooth muscle hypertrophy, we found a decrease in neuronal NOS activity in the rat bladder (16). This decrease in NO production may promote the dedifferentiation and phenotypic changes of the smooth muscle demonstrated after bladder outlet obstruction.

In this study, we demonstrated that iNOS is preferentially expressed in dedifferentiated BSMC and that actin filament dynamics and Rho signaling regulate cytokine-induced iNOS expression in BSMC. This may have implications during pathological conditions in the bladder where phenotypic changes and tissue remodeling are observed. Phenotypic changes and impairment in actin cytoskeleton formation may potentiate cytokine activation and in turn increase NO production in the bladder during disease.

GRANTS
This project was supported by the Swedish Medical Research Council (12601, AB694) and the Foundations of Crafoord and Magnus Bergvall. R. Johansson was financed by The Swedish Foundation of Strategic Research.
REFERENCES


