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RESEARCH PAPER

Irsogladine maleate potentiates the effects of nitric oxide on activation of cAMP signalling pathways and suppression of mesangial cell mitogenesis

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Background and purpose: Deficiency in nitric oxide (NO) is a major factor leading to deterioration and progression of certain glomerular diseases. Agents enhancing NO availability and potentiality are renoprotective. Irsogladine maleate (IM), an anti-ulcer drug, is reported to improve gastric blood flow via NO-dependent mechanisms. We, therefore, asked whether and how IM interacted with NO on glomerular mesangial cells.
Experimental approach: Mesangial cells were exposed to IM and NO donors. Activation of cAMP signalling pathways was assessed by intracellular cAMP, phosphorylation of VASP, activation of the cAMP response element (CRE) and expression of CRE-regulated proteins.
Key results: IM alone did not affect cell proliferation. However, it greatly enhanced the growth-inhibitory effect of NO donor S-nitroso-N-acetylpenicillamine (SNAP). IM acted synergistically with NO on suppression of mitogen-activated protein kinase activation, induction of gap junction protein connexin43, increase of intracellular cAMP, and phosphorylation of VASP. With the use of the CRE-SEAP-based reporting system, IM and SNAP cooperatively activated cAMP response elements (CRE). A similar activation of cAMP was induced by IM with two different NO donors, the sGC activator Bay 41-2272 and the cGMP analogue 8-bromo-cGMP. The effects of SNAP and IM on cAMP activation were mimicked by phosphodiesterase 3 (PDE3) and PDE4 inhibitors. In addition, IM markedly augmented cytokine-induced expression of iNOS, production of NO and activation of CRE.
Conclusion and implications: The effects of NO were greatly potentiated by IM through synergistic activation of cAMP pathway. Combined therapy with IM and NO may be developed for certain renal diseases.
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Keywords: mesangial cell; irsogladine maleate; nitric oxide; phosphodiesterase; cAMP; proliferation; cAMP response element; connexin43

Abbreviations: 8-Br-cGMP, 8-bromo-cGMP; Cx43, connexin43; CRE, cAMP response element; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-1β, interleukin-β; iNOS, inducible nitric oxide synthase; LDH, lactate dehydrogenase; L-NAME, N^G-nitro-L-arginine methylester; MAP kinase, mitogen-activated protein kinase; NONOate, (Z)-1-[-2-(aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate; ODQ, 1*H*-[1,2,4]oxadiazolo[4, 3-α-]quinoxalin-1-1; 8-pCPT-cGMP, 8-(4-chlorophenylthio)-guanosine 3′,5′-cyclic monophosphate; PDE, phosphodiesterase; PDGF, platelet-derived growth factor; PKA, protein kinase A; PKG, protein kinase G; RP-cGMP, Rp-8-pCPT-cGMP; SEAP, secreted alkaline phosphatase; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SNP, sodium nitroprusside; TNF-α, tumor necrosis factor-α; VASP, vasodilator-stimulated phosphoprotein

Introduction

Mesangial cells are specialized smooth muscle cells. Situated at the center of glomerulus, mesangial cells play key roles in glomerular pathophysiology. Under normal condition, mesangial cells maintain a quiescent phenotype; few cells undergo cell proliferation. However, in the glomerular diseases, proliferation of mesangial cells is one of key events

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in the pathogenesis of glomerular injury. Many factors have been identified as regulating mesangial cell growth. Among them, vasoconstrictive substances and growth factors, such as angiotensin II, endothelin and platelet-derived growth factor (PDGF), are potent mitogens, whereas the vasodilator nitric oxide (NO) and agents elevating intracellular cAMP and/or cGMP are strong inhibitors of mesangial cell proliferation.

NO is an important molecule with multiple biological effects. Besides its vasodilating actions, NO regulates mesangial cell growth, migration, adhesion, matrix production and apoptosis (Garg and Hassid, 1989; Muhl *et al*., 1996; Craven *et al*., 1997; Yao *et al*., 1998; Rupprecht *et al*., 2000). Under physiological conditions, low concentrations of NO, released by glomerular endothelial cells, plays a pivotal role in control of glomerular hemodynamics and maintenance of quiescent mesangial cell phenotype. Under pathological conditions, NO generation in the glomeruli is markedly enhanced due to the induction of NO synthase expression in intrinsic glomerular cells and infiltrating, activated macrophages. Activation of the NO system may either protect against or contribute to progression of inflammatory renal diseases (Narita *et al*., 1995; Ishizuka *et al*., 2000). In non-inflammatory renal diseases, a deficiency in NO, subsequent to the dysfunction of endothelial cells, is one of the major factors leading to the irreversible glomerular lesions (Jover and Mimran, 2001; Kang *et al*., 2002). Agents enhancing NO availability and function have been experimentally demonstrated to be renoprotective in a variety of experimental renal diseases, particularly those characterized by derangements of glomerular hemodynamics (Noris and Remuzzi, 1999; Klahr and Morrissey, 2004; Wang *et al*., 2005). However, therapeutic utility of these agents in the clinic has been limited, due to the potential unfavorable effects and the necessity for preclinical trials. It would be a great advantage to use drugs that have already been proved safe in clinical practice, to potentiate the biological activities of NO.

Irsogladine maleate (IM) [2,4-diamino-6-(2,5-dichlorophenyl)-*s*-triazine maleate] is an anti-ulcer drug with few side effects. The therapeutic mechanisms of IM could be related to the improvement of mucosal blood flow (Yamamoto *et al*., 1999; Kyoi *et al*., 2003) and augmentation of gastric epithelial barrier functions (Iwata *et al*., 1998; Takahashi *et al*., 2000). In a rat model of gastric injury induced by monochloramine, a substance highly toxic to mucosal tissue, treatment with IM significantly improved the mucosal blood flow and decreased gastric lesions. This effect of IM was NO dependent. Blockade of inducible nitric oxide synthase (iNOS) using a specific inhibitor N^G-nitro-L-arginine methylester (L-NAME) completely abolishes the protective effects of IM (Kyoi *et al*., 2003). Similar results have been obtained by Yamamoto *et al*. (1999). These observations indicate that IM has close interactions with NO. Although the molecular events linking IM to NO remain to be clarified, the NO dependency of the therapeutic effect of IM suggests that IM may be exploited to regulate the biological activities of NO.

IM is also known to protect gastric mucosal barrier functions through induction of gap junction (GJ) protein expression and promotion of intercellular GJ communication (Iwata *et al*., 1998; Takahashi *et al*., 2000). IM induces expression of several different GJ proteins, including connexin26, 32 and 43. This effect of IM is mediated by cAMP signaling (Nakashima *et al*., 2000; Kawasaki *et al*., 2002; Uchida *et al*., 2005). More recently, IM has been characterized as an inhibitor of phosphodiesterase 4 (PDE4) (Kyoi *et al*., 2004a, b), a predominant cAMP-degrading enzyme present in various cell types (Beavo, 1995; Dousa, 1999). Interestingly, NO also increases GJ protein expression via protein kinase A (PKA)-dependent mechanisms. The effect of NO involves cGMP-mediated inhibition of PDE3 (Yao *et al*., 2005). Given that the breakdown of cAMP is predominantly catalyzed by PDE3 and PDE4 in a variety of cell types, including mesangial cells (Beavo, 1995; Matousovic *et al*., 1995; Dousa, 1999; Zhu *et al*., 2006), IM and NO may synergistically activate cAMP signaling pathways via combined inhibition of PDE3 and PDE4. If so, the therapeutic efficacy of these agents will be greatly potentiated. The objective of the present study was to test this hypothesis.

In this current study, a synergy between IM and NO in activation of cAMP signaling pathways and in suppression of mesangial cell mitogenesis is revealed. Combined therapy with IM and NO donors could be developed for treatment of certain renal diseases.

Materials and methods

Establishment of reporter cells

Clonal mesangial cells (SM43) were established from isolated renal glomeruli of a male Sprague–Dawley rat and identified as being of the mesangial cell phenotype as described before (Kitamura *et al*., 1994). Cells were maintained in Dulbecco's modified Eagle's medium/F-12 (DMEM/F-12; Gibco-BRL, Gaithersburg, MD, USA) supplemented with 100 U ml^{-1} penicillin G, 100 μg ml^{-1} streptomycin, 0.25 μg ml^{-1} amphotericin B and 5% fetal bovine serum (FBS). Using a calcium–phosphate co-precipitation method, SM/cAMP response element (CRE)-secreted alkaline phosphatase 15 (SEAP15) cells were established by transfection of SM43 cells with pCRE-SEAP (BD Biosciences, Palo Alto, CA, USA) together with pcDNA3.1 (Invitrogen, Carlsbad, CA, USA), as described previously (Yao *et al*., 2005, 2006; Zhu *et al*., 2006). pCRE-SEAP encodes SEAP under the control of three copies of CRE. Medium containing 0.5% FBS was generally used for studies.

Western blot analysis

Extracted cellular proteins were separated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels and electrotransferred onto polyvinylidine difluoride membranes. After blocking with 3% bovine serum albumin in phosphate-buffered saline (PBS), the membranes were incubated with anti-vasodilator-stimulated phosphoprotein (VASP) antibody (dilution 1:1000; Chemicon International, Temecula, CA, USA), anti-connexin43 (Cx43) antibody (dilution 1:2000; Sigma, St Louis, MO, USA), anti-phospho-mitogen-activated protein (MAP) kinase antibody (dilution 1:1000; Cell Signaling, Danvers, MA, USA) or anti-iNOS antibody (dilution 1:1000, Lab Vision, Fremont CA, USA). After washing with

PBS containing 0.1% Tween 20, the filters were probed with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG, and the bands were visualized by the enhanced chemiluminescence system (Amersham Biosciences, Buckinghamshire, UK). To confirm equal loading of proteins, the filters were soaked in 62.5 mM Tris-HCl (pH 6.8) containing 2% SDS and 100 mM β-mercaptoethanol for 30 min at 60°C and reprobed with monoclonal anti-β-actin antibody (dilution 1:30 000; Sigma) or anti-extracellular signal-regulated kinase (ERK) antibody (dilution 1:1000; Cell Signaling).

Northern blot analysis
Total RNA was extracted by a single-step method, and Northern blot analysis was performed as described earlier (Yao *et al*., 2005). cDNAs for iNOS, SEAP and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used to prepare radiolabeled probes.

SEAP assay
Activity of SEAP was evaluated using the Great EscAPe Detection Kit (BD Biosciences) following the protocol provided by the manufacturer. In brief, reporter cells in 96-well plates were exposed to stimuli for the indicated time periods, and culture media were collected and centrifuged at 12 000 *g* for 2 min. Fifteen microliters of dilution buffer was mixed with 5 μl of the sample, and the mixture was incubated at 65°C for 30 min to eliminate endogenous alkaline phosphatase activity. Twenty microliters of assay buffer were subsequently added to the mixture and incubated for additional 10 min at room temperature. CSPD chemiluminescence substrate was diluted with 20 × chemiluminescence enhancer to 1.25 mM, and 20 μl of the diluted substrate was added to each sample, followed by 10 min incubation at room temperature. The intensity of chemiluminescent signal was determined by a luminometer (Gene Light 55; Microtech Nition, Chiba, Japan). Assays were performed in quadruplicate. SEAP activity was either expressed as fold induction against control or relative light unit.

Assessment of cAMP
Confluent mesangial cells in 24-well culture plates were treated with IM, *S*-nitroso-*N*-acetylpenicillamine (SNAP) or both for 1 h, and the cells were lysed and assayed for cAMP using cAMP Biotrak Enzymeimmunoassay System (Amershan Biosciences) according to the instructions provided by the manufacturer.

Assessment of cell growth
Mesangial cell proliferation was measured using a tritium thymidine (^{3}H-TdR) incorporation assay. Cells were plated in a 96 well at a density of 10 000 cells per well in DMEM/F12 containing 0.5% FCS for 48 h. After that cells were exposed to various stimulus for an additional 24 h. ^{3}H-TdR (2 μCi well^{-1}) was added 16 h before cell harvesting. The cells were collected on a filter by using an automated cell harvester. Radioactive thymidine incorporation was measured by scintillation counting.

Cytotoxicity assay
Cytotoxicity was evaluated by the release of lactate dehydrogenase (LDH) using an LDH cytotoxicity detection kit (Takara Bio Inc., Otsu, Shiga, Japan), as described by Zhu *et al*. (2006).

Measurement of nitrite levels
NO production was assayed by detecting nitrite accumulation in culture medium using the Griess reagent (Green *et al*., 1982). Briefly, 100 μl of a solution containing 1% sulphanilamide, 0.1% naphylethylenediamine in 2 M HCl was added to 100 μl of conditioned medium. Samples were incubated at room temperature for 10 min, and then their absorbance was measured with a microtiter plate-reader at 550 nm. Nitrite levels were expressed in nanomoles NO_2 per microgram of total cellular protein.

Statistical analysis
Assays were performed in quadruplicate, and data were expressed as means ± standard error of the mean (s.e.m.). Statistical analysis was performed using one-way analysis of variance and the Dunnett test. Comparison of two populations was performed by Student's *t*-test. $P<0.05$ was considered to show a statistically significant difference.

Reagents
Human PDGF-BB was obtained from Perro Tech Inc. (Rocky Hill, NJ, USA). Cilostamide and IM were purchased from Wako (Osaka, Japan). Human recombinant interleukin-1β (IL-1β) and human recombinant tumor necrosis factor-α (TNF-α) were generous gifts by Otsuka Pharmaceutical Co. Ltd (Tokushima, Japan) and Dr Katsuo Noguchi (Teikyo University School of Medicine, Tokyo, Japan), respectively. All other reagents were purchased from Sigma (St Louis, MO, USA).

Results

Suppression of mesangial cell mitogenesis by IM and NO
First we examined the effects of IM and the NO donor, SNAP, on mesangial cell proliferation. DNA synthesis was measured by incorporation of ^{3}H-TdR into mesangial cells. As shown in Figure 1a, incubation of mesangial cells with SNAP led to concentration-dependent inhibition of PDGF-induced mesangial cell proliferation. SNAP at concentrations over 10 μM significantly inhibited cell proliferation. In contrast, 10 μM IM did not affect mesangial cell proliferation (Figure 1a and b). However, it significantly potentiated the anti-proliferative effect of SNAP (Figure 1a). This effect of IM was observed at the concentration as low as 1 μM. Besides SNAP, IM similarly amplified the growth inhibition induced by another NO donor (Z)-1-[-2-(aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (NONOate) (Figure 1b). Of note, IM and SNAP, either alone or in combination, did not exhibit any cytotoxic effects at the concentrations tested, when evaluated by release of LDH (see Supplementary Figure S1).

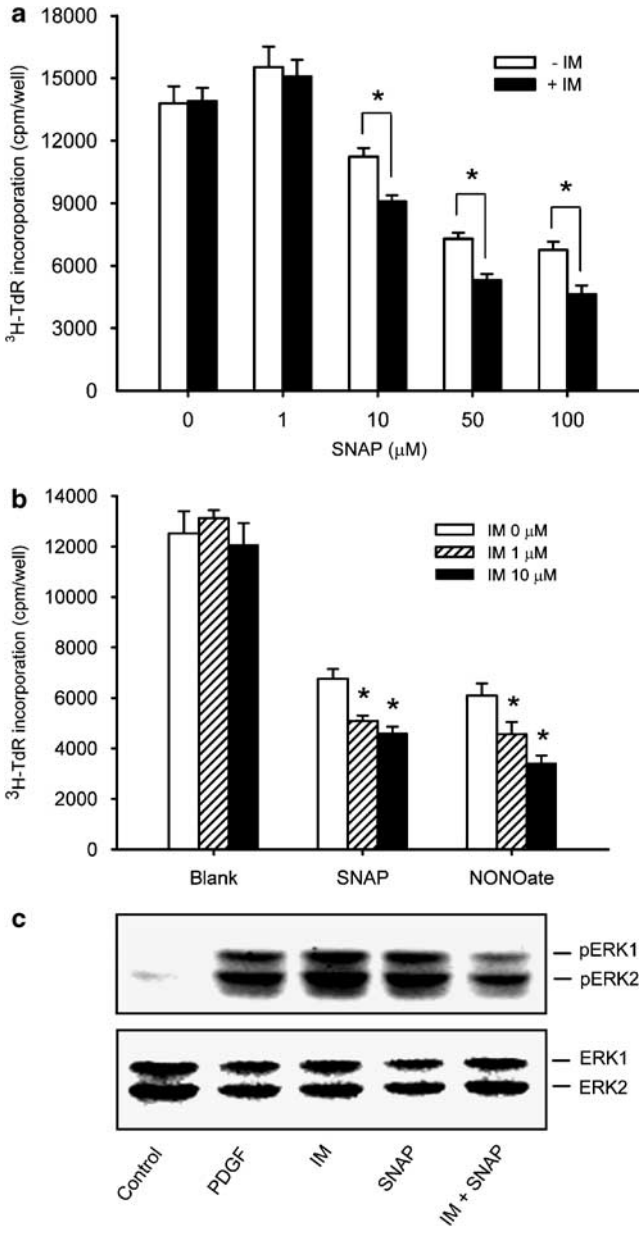

Figure 1 Suppression of mesangial cell proliferation by IM and SNAP. Mesangial cells in 96-well plates under the stimulation of 20 ng ml^{-1} PDGF-BB were exposed to different concentrations of SNAP (**a**) or IM (**b**) alone or in combination with 10 μM IM (**a**) or 100 μM SNAP or 100 μM NONOate (**b**) for 24 h. Cell proliferation was evaluated by thymidine (^{3}H-TdR) incorporation. Asterisks indicate statistically significant differences; *$P<0.01$ versus SNAP or NONOate alone. (**c**) Inhibition of PDGF-induced activation of ERK by IM and SNAP. Mesangial cells were pretreated with 10 μM IM, 100 μM SNAP or IM plus SNAP for 1 h, and exposed to 20 ng ml^{-1} PDGF for 5 min. Cellular proteins were subjected to Western blot analysis for phosphorylated ERK1 (pERK1) and ERK2 (pERK2). Protein levels of ERKs were shown at the bottom as loading controls.

MAP kinase activation is an early signaling event leading to cell proliferation. PDGF is a well-known mitogen that triggers the activation of MAP kinase in mesangial cells (Shultz *et al*., 1988). We, therefore, examined the effect of IM and SNAP on MAP kinase activation. As shown in Figure 1c, PDGF markedly induced phosphorylation of ERK1 and ERK2. Treatment of the cells with 10 μM IM plus 100 μM SNAP clearly attenuated PDGF-induced activation of ERKs.

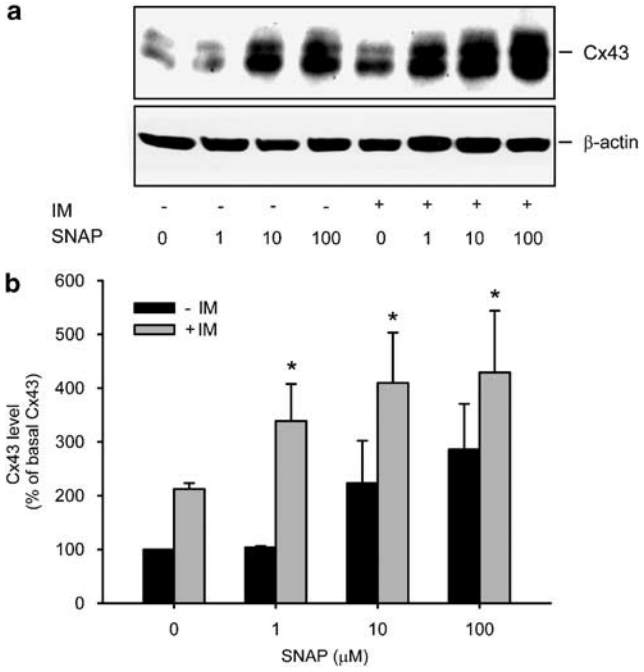

Figure 2 Induction of Cx43 by IM and SNAP. Mesangial cells were treated with 10 μM IM, 100 μM SNAP or IM plus SNAP for 24 h. The cellular proteins were subjected to Western blot analysis for Cx43. The level of β-actin was shown as a loading control (**a**). Densitometric analysis of the data is shown in (**b**). Asterisks indicate statistically significant difference (*$P<0.05$ versus SNAP alone). The blot shown is representative of three separate experiments with similar results.

Cooperative induction of Cx43 expression by IM and NO

Given that the levels of GJ proteins are reversibly correlated with cell proliferation (Goodenough *et al*., 1996; Kumar and Gilula, 1996) and that both IM and NO are known stimulators of connexin Cx43 expression (Nakashima *et al*., 2000; Kawasaki *et al*., 2002; Yao *et al*., 2005), we therefore examined the induction of Cx43 by these agents in mesangial cells. As shown in Figure 2, incubation of mesangial cells with SNAP caused a concentration-dependent elevation in Cx43 level. This effect of SNAP was greatly potentiated in the presence of 10 μM IM. IM itself also modestly elevated Cx43 expression.

Synergistic activation of cAMP signaling pathways by IM and NO

Previous studies documented that the effects of IM and NO on Cx43 levels are mediated by cAMP signaling (Nakashima *et al*., 2000; Kawasaki *et al*., 2002; Yao *et al*., 2005). The cooperative induction of Cx43 suggests that IM and NO may additively activate cAMP signal pathway. To confirm this, we measured the intracellular cAMP. Figure 3a shows that SNAP (100 μM) or IM (10 μM) alone elicited a small, but statistically significant increase in cAMP and, in combination, they synergistically elevated cAMP.

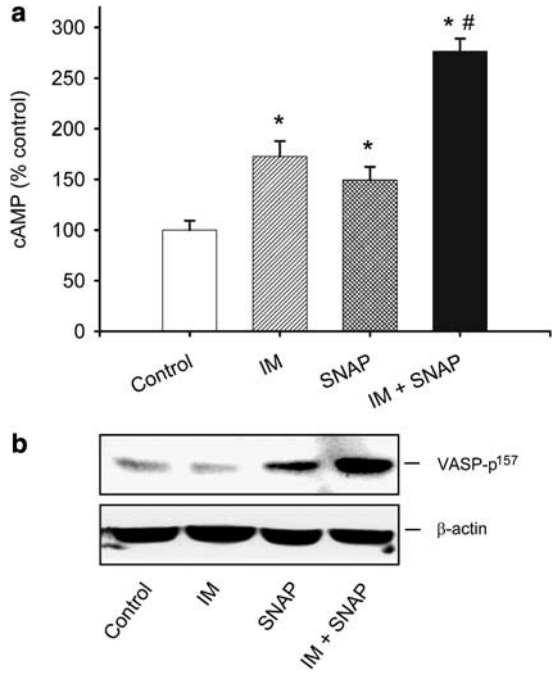

Figure 3 Elevation of intracellular cAMP and activation of PKA by IM and SNAP. (**a**) Effects of IM and SNAP on the level of cAMP. Mesangial cells were treated with 10 μM IM and 100 μM SNAP, alone or in combination, for 1 h, and cellular extracts were subjected to assays for intracellular cAMP. $^{*}P<0.01$ versus control; $^{\#}P<0.01$ versus single stimulus; mean$\pm$s.e.m.; $n=4$. (**b**) Phosphorylation of VASP, an indicator for PKA activation, by IM and SNAP. Mesangial cells were treated with 10 μM IM, 100 μM SNAP or IM plus SNAP for 1 h, and cellular proteins were subjected to Western blot analysis for phosphorylated VASP at serine 157. The level of β-actin was shown as a loading control.

In parallel with the increase in cAMP, PKA activation was also potently induced by IM and SNAP. This was reflected by the phosphorylation level of a PKA substrate VASP at serine 157 (Figure 3b).

Increase in cAMP causes PKA activation and subsequently activates CRE, leading to expression of genes that have CRE in their regulatory regions. As an event downstream of PKA activation, CRE activity serves as a reliable indicator for evaluating the status of cAMP signaling pathways (Zhu *et al*., 2006). We therefore analyzed the cAMP-elevating effects of IM and SNAP by using a CRE-SEAP-based reporting system (Zhu *et al*., 2006). Incubation of reporter cells with IM or SNAP did not greatly alter CRE activity. However, when used in combination (Figures 4a and b), they elevated CRE activity markedly, in a manner depending upon both the concentrations of SNAP and IM (SEAP activity: control, 1 ± 0.08; 10 μM IM, 0.87 ± 0.11; 100 μM SNAP, 1.38 ± 0.04; IM plus SNAP, 5.45 ± 0.20; mean$\pm$s.e.m.; $n=4$). Time-lapse experiments revealed that the significant induction of SEAP activity by IM and SNAP was observed 6 h after the stimulation (SEAP activity: control, 620 ± 42; 10 μM IM, 666 ± 30; 100 μM SNAP, 755 ± 37, IM plus SNAP 1843 ± 66; mean$\pm$s.e.m., $n=4$). The difference between single and combined stimulation on CRE activation persisted throughout the experimental period (30 h) (Figure 4c).

Activation of CRE was also induced by treatment of cells with IM and other structurally different NO donors, including sodium nitroprusside (SNP) and NONOate (Figure 4d). This result suggests that NO, but not other metabolites, is involved in synergy with IM in activation of cAMP signal.

Besides exogenous NO, the effects of endogenous NO were also potentiated by IM. As shown in Figure 4e, stimulation of the cells with the cytokines (10 ng ml^{-1} TNF-α plus 1 ng ml^{-1} IL-1β) that induces endogenous NO formation, did not affect CRE activity. However, in the presence of 10 μM IM, a significant elevation in SEAP activity was observed. This elevation could be abolished by the iNOS inhibitor L-NAME (1 mM) or the soluble guanylate cyclase (sGC) inhibitor 1*H*-[1,2,4]oxadiazolo[4,3-α-]quinoxalin-1-1 (ODQ; 10 μM).

Activation of CRE by IM and SNAP was also confirmed by mRNA expression of the reporter gene SEAP (Figure 4f).

Evidence for the combined inhibition of PDE3 and PDE4 in the synergistic effect of NO and IM

NO activates cAMP signaling pathway via cGMP-mediated inhibition of PDE3 (Osinski *et al*., 2001; Aizawa *et al*., 2003; Yao *et al*., 2005). On the other hand, IM elevates cAMP via suppression of PDE4 activity (Kyoi *et al*., 2004a, b). Given that PDE3 and PDE4 are two major degrading enzymes for cAMP in mesangial cells (Matousovic *et al*., 1995; Dousa, 1999; Zhu *et al*., 2006), the synergistic activation of cAMP by NO and IM could be a result of the combined inhibition of PDE3 and PDE4. To test this hypothesis, we assessed the mediating role of cGMP. As shown in Figure 5a, activation of CRE by SNAP and IM could be completely blocked by the inhibitor of sGC, ODQ (10 μM), but was not affected by the protein kinase G (PKG) inhibitor, Rp-8-Br-PET-cGMP (RP-cGMP; 100 μM). In addition, the role of SNAP was mimicked by an activator of sGC, Bay 41-2272 (10 μM) and a cGMP analog, 8-bromo-cGMP (8-Br-cGMP) (500 μM). Another cGMP analog, pCPT-cGMP (500 μM), which activates PKG, but does not interact with PDE3 (Osinski *et al*., 2001; Aizawa *et al*., 2003), did not display any synergistic effect with IM on CRE activation (Figure 5b). The difference between 8-Br-cGMP and pCPT-cGMP in cooperation with IM in activation of CRE was not due to differences in their ability to activate PKG. Both agents activated PKG to an extent comparable to SNAP (Figure 5c), as evaluated by the phosphorylation level of VASP at serine 239, a PKG-preferred phosphorylation site (Oelze *et al*., 2000; Ibarra-Alvarado *et al*., 2002).

If the effects of SNAP and IM are mediated by inhibition of PDE3 and PDE4, one would expect that PDE3 and PDE4 inhibitors should be able to reproduce the effects. Figure 6a shows that the respective roles of SNAP and IM in CRE activation were substituted by PDE3 and PDE4 inhibitors. In the presence of SNAP, PDE4 inhibitor rolipram (20 μM) substantially activated CRE. Similarly, in the presence of IM, enhanced activation of CRE by SNAP was reproduced by the treatment of cells with PDE3 inhibitor cilostamide (20 μM). In addition, PDE3 and PDE4 inhibitors together induced a striking increase in CRE activity. On the other hand, no super-additive effects on CRE activity were found when IM was added in combination with rolipram or SNAP with cilostamide (Figure 6b).

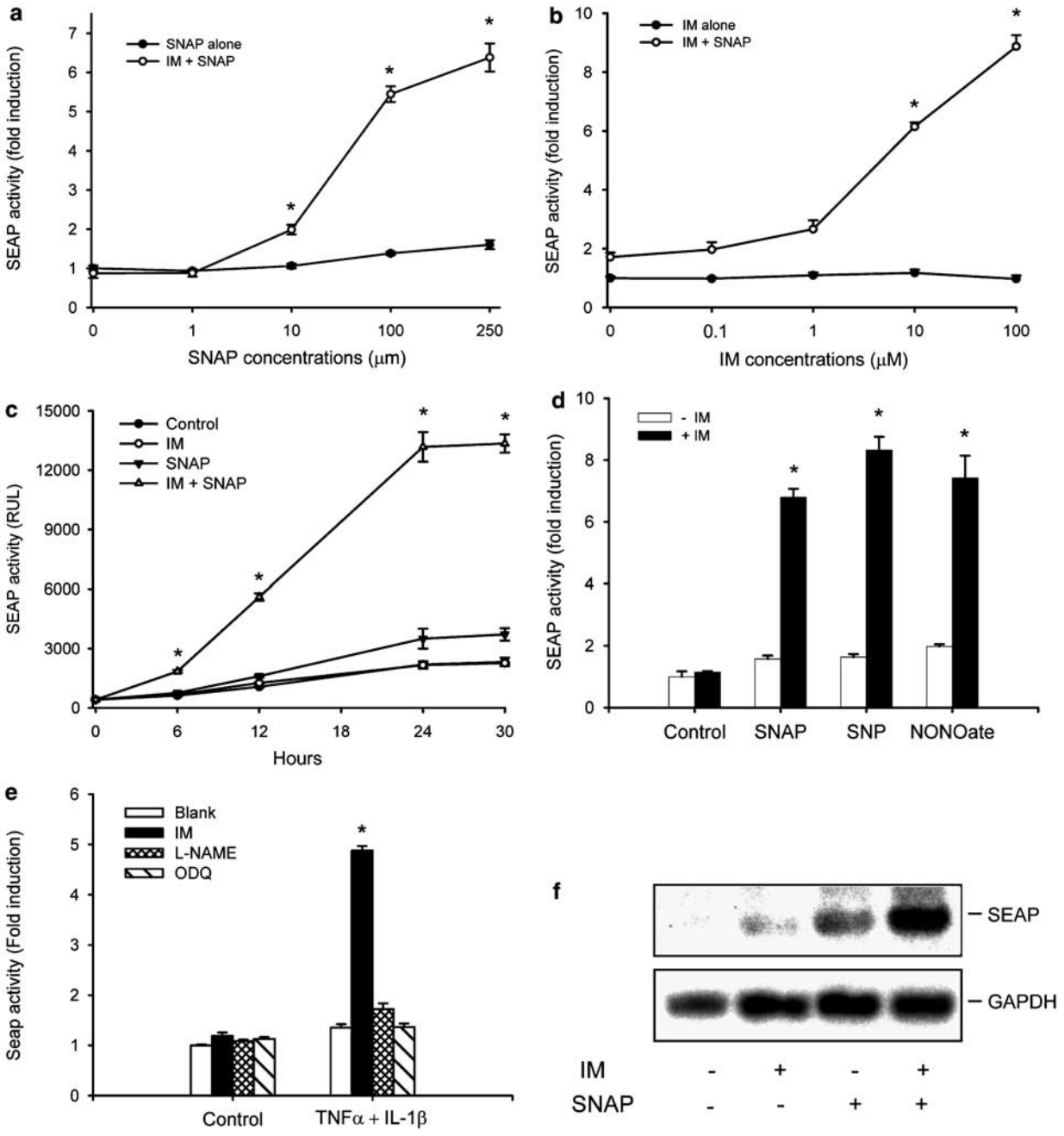

Figure 4 Activation of CRE by IM and SNAP. (**a**) Potentiation of SNAP-elicited activation of CRE by IM. Mesangial cells that were stably transfected with pCRE-SEAP were exposed to the indicated concentration of SNAP in the presence or absence of 10 μM IM for 24 h. The conditioned media were harvested and assayed for SEAP activity. Asterisks indicate statistically significant differences (*$P<0.01$ versus control). (**b**) Enhancement of the action of IM by SNAP. The reporter cells were treated with indicated concentration of IM alone or together with 100 μM SNAP for 24 h. (**c**) Time-dependent induction of CRE activation by IM and SNAP. The reporter cells were treated with IM, SNAP, or IM plus SNAP for the indicated time period. (**d**) Cooperative activation of CRE by IM and different NO donors. The reporter cells were treated with 100 μM SNAP, 200 μM SNP, or 100 μM NONOate alone or in combination with 10 μM IM for 24 h. *$P<0.01$ versus basal control and single stimulus. (**e**) Synergy between endogenous NO and IM in induction of CRE activation. The reporter cells were either left untreated (blank) or treated with 1 mM N^G-nitro-L-arginine methylester (L-NAME) or 10 μM ODQ for 30 min, and then exposed to 10 μg ml^{-1} TNF-α plus 1 ng ml^{-1} IL-1β in the presence or absence of 10 μM IM for 24 h. Asterisks indicate statistically significant differences; *$P<0.01$ versus control without IM; mean$\pm$s.e.m.; $n=4$. (**f**) Northern blot analysis of SEAP mRNA expression in reporter cells that were treated with IM or SNAP, alone or in combination, for 12 h (upper panel). Expression of GAPDH was used as a loading control (bottom panel).

Consistent with the change of SEAP activity, these agents in different combinations also induced activation of PKA, as shown by the phosphorylation level of a PKA substrate VASP at serine 157 (Figure 6c).

Induction of cytokine-induced expression of iNOS and formation of NO by IM

Augmentation of NO effect by IM via cooperative activation of cAMP signaling promoted us to ask whether IM also

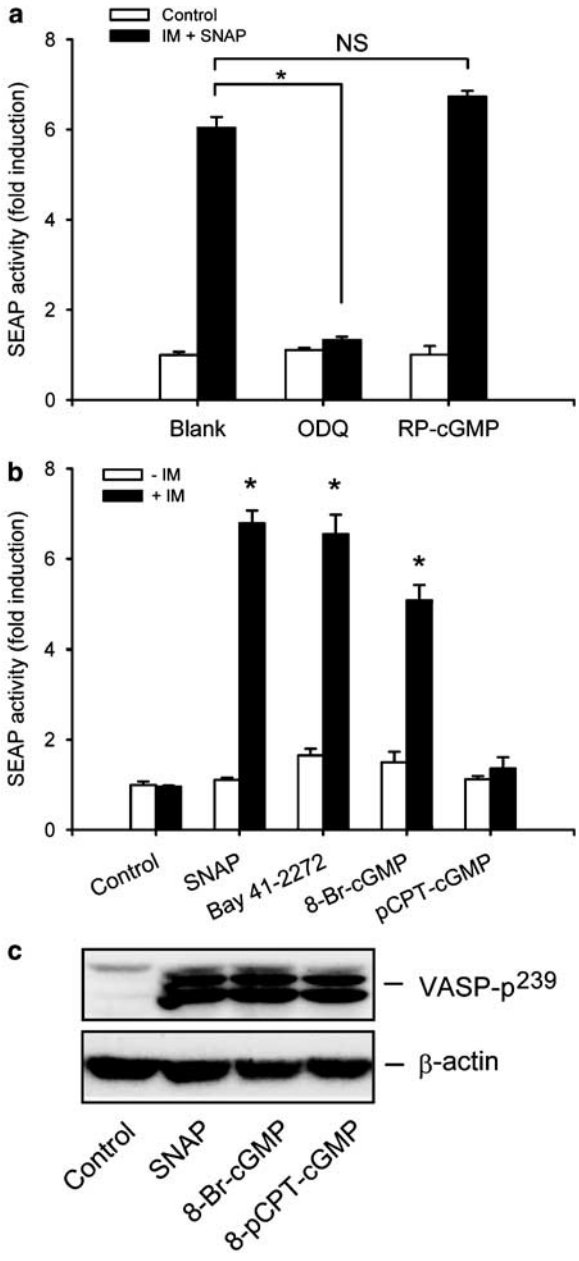

Figure 5 Involvement of cGMP in synergistic activation of cAMP signaling pathway by SNAP and IM. (**a**) Effects of the sGC inhibitor ODQ and the PKG inhibitor Rp-8-Br-PET-cGMP (RP-cGMP) on the activation induced by NO and IM. Mesangial cells were preincubated with 10 μM ODQ or 100 μM PKG inhibitor Rp-8-Br-PET-cGMP for 15 min before exposing to a mixture of SNAP (100 μM) and IM (10 μM) for 24 h in the presence of these agents. Asterisks indicate statistically significant differences (*$P<0.01$). NS, not significant. (**b**) Effects of IM and different cGMP analogs on CRE activation. Mesangial cells were treated with 100 μM SNAP, 10 μM Bay 41-2272, 500 μM 8-Br-cGMP, or 500 μM 8-pCPT-cGMP alone or in combination with 10 μM IM for 24 h. Asterisks indicate statistically significant differences (*$P<0.01$ versus basal control and single stimulus). (**c**) Phosphorylation of VASP at serine 239, an indicator for PKG activation, by SNAP and cGMP analogs. Mesangial cells were treated with 100 μM SNAP, 500 μM 8-Br-cGMP or 500 μM 8-pCPT-cGMP for 1 h, and cellular proteins were subjected to Western blot analysis for phosphorylated VASP at serine 239. The level of β-actin was shown as a loading control.

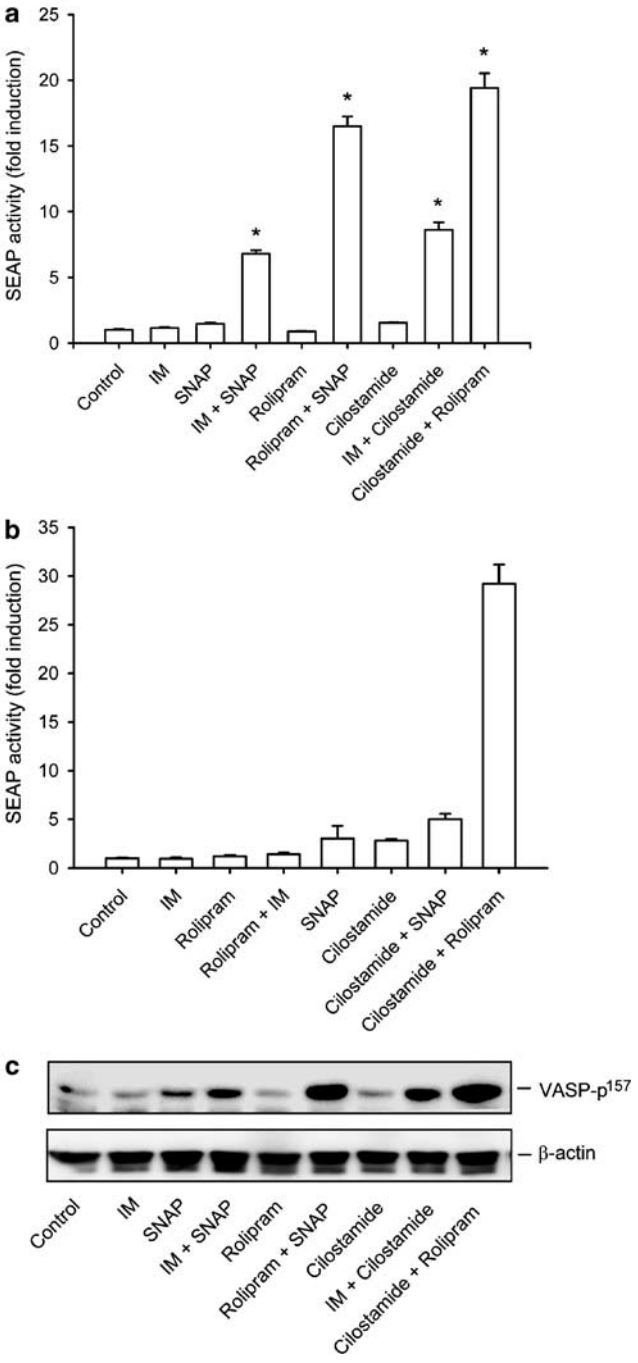

Figure 6 Reproduction of synergistic effects of SNAP and IM on cAMP signaling by PDE3 and PDE4 inhibitors. (**a** and **b**) PDE inhibitors on CRE activation. Mesangial cells were treated with 10 μM IM, 100 μM SNAP, 20 μM cilostamide, or 20 μM rolipram alone or in different combinations for 24 h. Asterisks indicate statistically significant differences (*$P<0.01$ versus basal control and single stimulus). (**c**) PDE inhibitors on PKA activation. Mesangial cells were treated with the indicated stimuli for 1 h, and cell proteins were subjected to Western blot analysis for phosphorylated VASP at serine 157.

affects the expression of iNOS and formation of NO. For this purpose, the influence of IM on the cytokine (10 ng ml^{-1} TNF-α plus 1 ng ml^{-1} IL-1β)-induced expression of iNOS was examined by using Northern and Western blot. Figure 7a

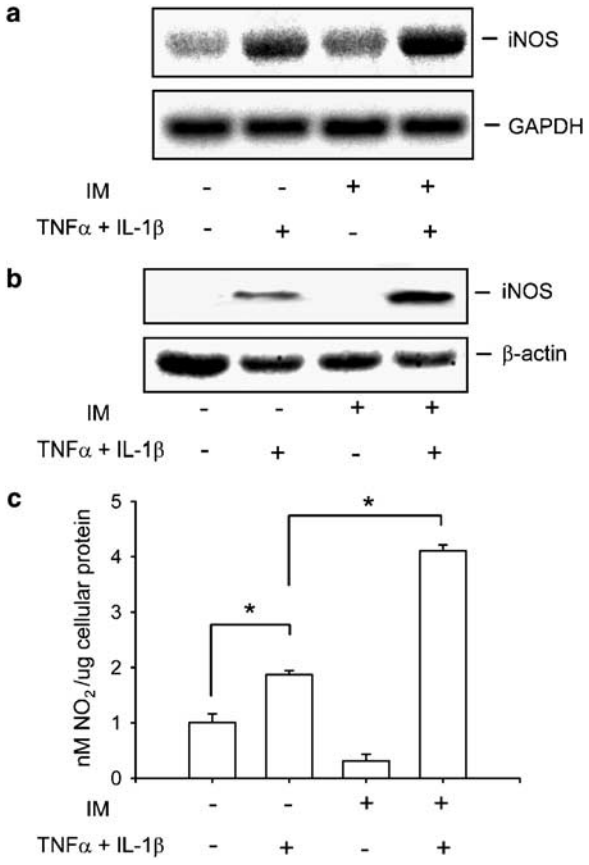

Figure 7 Potentiation of cytokine-elicited inducible NO synthase (iNOS) expression and NO formation by IM. Mesangial cells were treated with 10 ng ml^{-1} TNF-α plus 1 ng ml^{-1} IL-1β in the presence or absence of 10 μM IM. The expression of iNOS at mRNA and protein levels were analyzed by using Northern (**a**) and Western blot (**b**), respectively. Expression of GAPDH (**a**) and β-actin (**b**) was used as loading control. The conditioned media were harvested at 24 h for measurement of nitrite levels (**c**). Asterisks indicate statistically significant differences (*$P<0.01$; mean$\pm$s.e.m.; $n=4$).

and b shows that IM markedly increased iNOS expression at both mRNA and protein levels. Accordingly, the cytokine-induced formation of NO was also significantly augmented by IM as evaluated by the accumulation of nitrate production in the culture medium (Figure 7c).

Discussion

In this study, we found that NO and a gastroprotective drug, IM, when used in combination, synergistically elevated intracellular cAMP, activated PKA and CRE, induced expression of the CRE-regulated protein Cx43 and suppressed cell proliferation. Additionally, IM markedly enhanced cytokine-induced iNOS expression and NO formation.

Intracellular cAMP is raised by increased synthesis via activation of adenylyl cyclase and/or decreased degradation via inhibition of PDEs (Beavo, 1995; Dousa, 1999). Given that both NO and IM are known to affect PDE activities (Aizawa *et al*., 2003; Kyoi *et al*., 2004a, b; Yao *et al*., 2005), inhibition of PDEs could be the mechanism by which IM and NO synergistically elevated intracellular cAMP. NO exerts multiple effects on mesangial cells and many of them are mediated by PKG activation following cGMP generation. The effects of NO also involve modulation of cAMP signaling pathways via cGMP-mediated inhibition of PDE3 (Osinski *et al*., 2001; Aizawa *et al*., 2003; Yao *et al*., 2005). In this study, we demonstrated that the cooperative activation of cAMP signaling pathways was completely inhibited by the sGC inhibitor ODQ, but not by the PKG inhibitor Rp-8-bromo-PET-cGMP. This result indicates that the effect of NO requires generation of cGMP, but not PKG activation. Consistent with this observation, a stable analog of cGMP, 8-Br-cGMP, mimicked the effect of NO, whereas another analog 8-(4-chlorophenylthio)-guanosine 3′,5′-cyclic monophosphate (8-pCPT-cGMP), which selectively activates PKG but does not interact with PDE3 (Osinski *et al*., 2001), had no effect. Thus the effect of NO was most probably due to the cGMP-mediated inhibition of PDE3. Indeed, a specific PDE3 inhibitor cilostamide reproduced the effect of NO. On the other hand, IM has been reported to elevate intracellular cAMP via inhibiton of PDE4 (Kyoi *et al*., 2004a, b), a major cAMP-degrading enzyme, which accounts for two-thirds of the high-affinity cAMP-hydrolyzing activity in mesangial cells (Matousovic *et al*., 1995). Interestingly, although IM elevated cAMP to an extent comparable to that achieved by SNAP, it only marginally inhibited mesangial cell proliferation. This is, in fact, in good agreement with the characteristic of PDE4. Previous studies have indicated that inhibition of PDE4 does not greatly affect cell proliferation, which has been explained by the compartmentalization of cAMP pools in mesangial cells (Dousa, 1999; Cheng *et al*., 2004). The PDE4-regulated cAMP pools are less effective in suppression of the growth-related Ras-Raf-MAP kinase signals (Cheng *et al*., 2004). The possible involvement of PDE4 inhibition in the cooperative effect was also supported by the fact that the PDE4 inhibitor rolipram similarly potentiated the effect of SNAP on activation of cAMP signaling pathways and in suppression of mesangial cell mitogenesis (data not shown). Of note, rolipram induced a much stronger activation of cAMP signals, in comparison with IM. The discrepancy could be caused by the different potency of these agents as inhibitors of PDE4. Taken together, our results thus support the idea that the combined inhibition of PDE3 and PDE4 was the basis of the synergistic activation of cAMP signaling pathways by SNAP and IM, as shown in the scheme in Figure 8.

Suppression of mesangial cell mitogenesis by NO may also involve the activation of a cAMP pathway via cGMP-mediated inhibition of PDE3. Both cGMP-dependent and independent mechanisms have been proposed for NO-induced growth inhibition. Rupprecht *et al*. (2000) demonstrated that NO inhibits mesangial cell proliferation through nitrosylation of the transcription factor Egr. Garg and Hassid (1989) reported that the growth-inhibitory effect of NO is mediated by cGMP. Molecular events downstream of cGMP have not been characterized in mesangial cells. In smooth muscle cells, Osinski *et al*. (2001) described that organic nitrates inhibit cell proliferation by activation of PKA via inhibition of PDE3. A similar involvement of cAMP signaling in NO-induced growth inhibition in mesangial cells is possible, because (1) cAMP-elevating agents are well-known

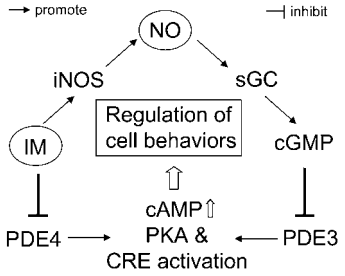

Figure 8 Schematic diagram illustrating potential mechanisms involved in the synergistic activation of cAMP signaling pathway by NO and IM. PDE3 and PDE4 are two major cAMP-degrading isoenzymes in mesangial cells. IM inhibits PDE4. On the other hand, NO activates sGC, causing the generation and action of cGMP. Combined inhibition of PDE3 and PDE4 by cGMP and IM results in increased intracellular cAMP, activation of PKA and induction of CRE activity, leading to changes in cell behaviours (such as Cx43 expression and growth inhibition). The synergistic activation of cAMP signaling is further augmented by IM-elicited expression of iNOS and production of NO.

inhibitors of mesangial cell mitogenesis (Matousovic *et al*., 1995; Dousa, 1999; Cheng *et al*., 2004); (2) inhibition of PDE3 by specific inhibitors results in significant suppression of mesangial cell mitogenesis (Matousovic *et al*., 1995; Dousa, 1999; Cheng *et al*., 2004); (3) the GJ protein, Cx43, the level of which is inversely related to cell growth (Goodenough *et al*., 1996; Kumar and Gilula, 1996), is induced by NO via cAMP (Yao *et al*., 2005); and (4) our current study demonstrates that suppression of mesangial cell mitogenesis by IM, and NO is positively correlated with their potency in activation of cAMP. Collectively, these findings support the mediating role of cAMP signaling in NO-induced growth inhibition. In general, the biological effects of cAMP and cGMP are similar. They take part in the control of a variety of cellular functions, including relaxation, migration, adhesion and survival. Amplification of NO-induced cAMP signaling by IM could be an effective approach to potentiate the multiple biological activities of NO.

Induction of the connexon, Cx43, by IM and SNAP could be closely correlated with their growth inhibitory effect. Regulation of cell growth by modification of GJ proteins has been extensively documented. In general, expression of GJ proteins is inversely related to cell proliferation (Goodenough *et al*., 1996; Kumar and Gilula, 1996). In mesangial cells, Cx43 may be involved in the suppression of mitogenesis (Yao *et al*., 2000, 2006). For example, short-term exposure of mesangial cells to PDGF caused rapid and reversible inhibition of gap junctional communication via phosphatidylinositol 3-kinase (Yao *et al*., 2000), which is also involved in PDGF-induced mitogenesis of mesangial cells (Choudhury *et al*., 1994). Of note, upregulation of Cx43 expression is documented to be closely related to the gastroprotective effects of IM (Iwata *et al*., 1998; Takahashi *et al*., 2000). Previous studies showed that inhibition of GJs weakens the barrier function, whereas maintenance of GJs protects gastric mucosal barrier functions (Iwata *et al*., 1998; Takahashi *et al*., 2000). In this context, cooperative induction of Cx43 by IM and NO in the gastrointestinal system may have therapeutic implications.

IM not only potentiated the biological activities of NO, but also its production. Mechanisms by which IM regulates cytokine-induced iNOS expression and NO formation could involve its cAMP-elevating effect. Super-additive induction of cytokine-induced expression of iNOS and production of NO by cAMP have been reported previously (Muhl and Pfeilschifter, 1995).

Our findings may have clinical implications. IM is a safe and effective gastroprotective drug. At therapeutic plasma concentrations, it augmented both the production and function of NO. These results indicate that IM could be used for treatment of certain renal diseases in which a deficiency of NO is present (Jover and Mimran, 2001; Kang *et al*., 2002). On the other hand, our study also suggests that in situations where NO contributes to deterioration and progression of the diseases (Narita *et al*., 1995), IM should be applied with care.

In conclusion, we revealed that the cAMP-elevating effects of IM and NO could be potentiated through mechanisms involving combined inhibition of different PDEs. The finding suggests that combined therapy with IM and NO donors may greatly improve the efficacy of these agents in treatment of certain renal diseases.

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Conflict of interest

The authors state no conflict of interest.

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