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Inducible nitric oxide synthase-dependent stimulation of PKGI and phosphorylation of VASP in human embryonic kidney cells

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Abstract

Inducible nitric oxide synthase (iNOS) production of nitric oxide (NO) has been mostly associated with so-called nitrosative stress or interaction with superoxide anion. However, recent investigations have indicated that, as for the other isoenzymes producing NO, guanylyl cyclase (GC) is a very sensitive target of iNOS activity. To further investigate this less explored signaling, the NO-cyclic guanosine 3′–5′-monophosphate (NO–cGMP)-induced vasodilator-stimulated phosphoprotein (VASP) phosphorylation on serine 239 was investigated in human embryonic kidney 293 cells (HEK cells). First, the expression and activity of α2 and β1 NO-sensitive GC subunits was determined by Western blot analysis, reverse transcription–polymerase chain reaction and NO donors administration. Then, the expression of a functional cGMP-dependent protein kinase I (PKGI) was verified by addition of 8-Br-cGMP followed by determination of phosphorylation of VASP on serine 239. Finally, iNOS activation of this signaling pathway was characterized after transfection of HEK cells with human iNOS cDNA. Altogether our data show that iNOS-derived NO activates endogenous NO-sensitive GC and leads to VASP phosphorylation in HEK cells.
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Keywords: cGMP; NO donors; NO-sensitive guanylyl cyclase; PKGI; VASP; iNOS

1. Introduction

Nitric oxide (NO), one of the smallest and most ubiquitous signaling molecules, is involved in a wide variety of biological processes and is produced in mammals by NO synthase (NOS), namely neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2) and endothelial NOS (eNOS or NOS3) [1]. NO has been described more than 30 years ago as ligand of the NO-sensitive guanylyl cyclase (NO-sensitive GC, EC 4.6.1.2), the enzyme that catalyzes the conversion of guanosine 5′-triphosphate to cyclic guanosine 3′–5′-cyclic monophosphate (cGMP) [2]. NO-sensitive GC is not the only, but certainly the most important target for NO. NO-sensitive GC consists of two different subunits, designated α and β, both required for catalytic activity [3,4]. Two α NO-sensitive GC subunits (α1 and α2) and two β subunits (β1 and β2) are generated by separate genes. Only two heterodimers have been shown at protein level: α1/β1 the most common and α2/β1 [3,4]. The mRNA for a variant of the α2 NO-sensitive GC subunit (α2i), which contains 31 additional amino acids, was recently identified in a number of tissues including human liver, colon, and endothelium [5]. This subunit can compete with the α2 NO-sensitive GC subunit for binding to β1 subunit and acts as a dominant negative inhibitor [5].

Increase of cGMP by NO via activation of NO-sensitive GC allows regulation of three groups of protein: the cyclic nucleotide-gated channels, the cGMP-dependent phosphodiesterases (PDE) and the cGMP-dependent protein kinases (PKG) [4]. Latter pathway was discovered by studying platelet aggregation and smooth muscles relaxa-

Abbreviations: cAMP, cyclic adenosine 3′–5′-monophosphate; cGMP, cyclic guanosine 3′–5′-monophosphate; DEA/NO, 2,2-diethyl-1-nitroso-oxyhydrasine; DETA/NO, (*Z*)-1-[(2-aminoethyl)-*N*-(2-ammonioethyl) amino] diazen-1-ium-1,2-diolate; GC, guanylyl cyclase; HEK cells, human embryonic kidney 293 cells; H89, *N*-[2-(methylamino) ethyl]–5-isoquinolene-sulfonamide; IBMX, 3-isobutyl-1-methylxanthine; iNOS, inducible nitric oxide synthase; L-NMA, N^G-monomethyl-L-arginine; NO, nitric oxide; ODQ, 1H-[1,2,4] oxadiazolo [4,3-a] quinoxylin-1-one; PDE, phosphodiesterase; PKGI, cGMP-dependent protein kinase I; PVP, polyvinylpyrrolidone; RT–PCR, reverse transcription–polymerase chain reaction; SNAP, *S*-nitroso-*N*-acetyl-D,L-penicillamine; VASP, vasodilator-stimulated phosphoprotein

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tion [3] but it appears involved in several other NO modulated processes such as gene expression [6–10]. Two types of PKG are found in mammalian cells, types I and II, which are the products of two different genes [11]. Type I is expressed as two isoforms, PKGIα and PKGIβ generated by alternative splicing of the type I gene [12]. NO-related effects have been mostly linked to stimulation of PKGI although stimulation of PKGII has also been detected [7,13]. Proteins phosphorylated in response to PKGI activation in vivo or in intact cells, include cGMP-dependent PDE, myosin light chain kinase, inositol (1,4,5)-triphosphate receptor, Na^+/K^+-ATPase, phospholamban, thromboxane and vasodilator-stimulated phosphoprotein (VASP) [3,4,14,15]. The latter is a protein widely expressed [15–17] and VASP serine 239 phosphorylation (VASP-P^{239}) has been shown to be a useful monitor for PKGI activity in intact cells [18,19].

GC activation by NO has been mostly investigated downstream of constitutive NOS (nNOS and eNOS) pathway. However, iNOS-dependent increase of cGMP has been demonstrated in bronchial epithelial cells transfected with iNOS cDNA [20], vascular smooth muscle cells exposed to interleukin-1, tumor necrosis factor-α or lipopolysaccharide [21] and NO-donors allowing chronic sustained delivery of NO such as DETA/NO [22]. The existence of such pathway may underlie iNOS signaling providing co-expression of the different signaling partners and is of potential interest to explain effects independent of NO oxidative derivative. To address this question, the functional interaction iNOS–cGMP–PKGI was investigated in human embryonic kidney cells known to express NO-sensitive GC [19,23].

2. Materials and methods

2.1. Cell culture

Human embryonic kidney 293 cells (HEK cells) were cultured at 37 °C, 3.5% of CO_2 in DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum and penicillin/streptomycin (all from Gibco Life Technologies). 53,000 cells/cm^2 were seeded and treated 48 h later with 8-Br-cGMP (Sigma), 8-Br-cAMP (Sigma), *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP, Alexis), 2,2-diethyl-1-nitroso-oxyhydrasine (DEA/NO) or (*Z*)-1-[(2-aminoethyl)-*N*-(2-ammonioethyl) amino] diazen-1-ium-1,2-diolate (DETA/NO, both kind gift of Dr. L.K. Keefer, National Cancer Institute at Frederick, USA), 1H-[1,2,4] oxadiazolo [4,3-a] quinoxylin-1-one (ODQ, Tocris, Anawa Trading) and *N*-[2-(methylamino) ethyl]-5-isoquinolene-sulfonamide (H89, Calbiochem).

2.2. Western blot analysis

Cellular extracts were obtained by direct collection of cells in cold Laemmli solution (62.5 mM HCl–Tris pH 6.8, 2% SDS, 8.7% glycerol, 0.02% bromophenol blue, 4% β-mercaptoethanol). Western blot analysis were performed as previously described [24,25]. Total protein was separated on 10% SDS–PAGE. Polyclonal anti-NO-sensitive GC (Cayman) diluted at 1:2000, anti-PKGI (kind gift of Dr. S.M. Lohmann, Würzburg University, Germany) diluted at 1:5000 and anti-actin (Sigma) diluted at 1:2000 were used. Monoclonal anti-VASP phosphorylated Ser^{239}16C2 (Alexis) was diluted at 0.25 μg/ml. This last antibody required a special blocking solution (3% BSA, 1% PVP, 1% PEG, 0.2% Tween 20, PBS 300 mM) and was prepared in blocking solution diluted 1:2 in washing solution (PBS containing 0.1% Tween 20). Detection was achieved by enhanced chemiluminescence (Amersham Pharmacia). The antibody used for NO-sensitive GC detection, is directed against conserved peptides sequences: EQARAQDGLKKRLGKLKAT and EDFYEDLDRFEENGTQDSR for α and β subunits, respectively. Therefore, extracts from mouse lung and human bladder smooth muscle cells (kind gift of Dr. P. Frey, Laboratory of Experimental Pediatric Urology, Lausanne University Hospital [26]) were used as positive control. For PKGI, human umbilical vein endothelial cells transfected with PKGI cDNA (kind gift of Dr. S.M. Lohmann [27]) was used. For VASP positive control, human platelets were obtained by Blood Transfusion Center of Lausanne University Hospital.

2.3. Isolation of total RNA and RT–PCR

Extraction of RNA using guanidium thiocyanate was performed as described [28]. First-strand cDNA was synthesized from 1 μg of total RNA using AMV reverse transcriptase (Promega). α1 NO-sensitive GC subunit was examined by specific primers [29] which give rise to a 488 bp fragment. α1 NO-sensitive GC cDNA (kind gift of Dr. F. Murad, University of Texas Houston Health Science Center, Houston, Texas) was used as positive control. α2 NO-sensitive GC subunit expression was analyzed by use of primers [29] which can amplify both known variants α2 and α2i. In preliminary experiments only a weak band representing α2i was detected after 40 cycles. Therefore, internal primers (5′-primer CATTGCTCTGATGGCCTTGA and 3′-primer CAGGCAATAACGTGGCATTC) obtaining a 145 bp fragment, were used in the following conditions: PCR with external primers was performed during 25 cycles at 94 °C 30 s, 60 °C 30 s and 72 °C 30 s. Then, the product of the external primers PCR was diluted 100-fold and used in a second PCR with the internal primers for 25 cycles at 94 °C 30 s, 55 °C 30 s and 72 °C 30 s. RNA from HL-60 cells, known to express α2 NO-sensitive GC subunit [5], was used as positive control. β1 NO-sensitive GC subunit was investigated using specific primers (5′-primer TGGAGAAGGAGCCATGAAGA and 3′-primer TCTGTTCGGCTTGTGAGGTT) which allow to obtain a 345 bp fragment (40 cycles at 94 °C 30 s, 55 °C

30 s and 72 °C 1 min). β1 GC cDNA (kind gift of Dr. F. Murad) was used as positive control. Except for α2, 40 cycles of amplification were performed. RT–PCR products were controlled on 2% agarose gel. Fragments obtained were gel purified (QIAquick® Gel Extraction Kit, Qiagen) and sequence was verified by sequencing.

2.4. cGMP assay

For cGMP level determination, cells were plated in multiwell culture dishes (10^5 cells/well). HEK cells were incubated for 10 min with 0.5 mM 3-isobutyl-1-methyl-xanthine (IBMX, Sigma) and in the presence or absence of NO donors and/or NO-sensitive GC inhibitor ODQ. Mock and iNOS transfected cells (see below) were seeded 72 h after the transfection and incubated 6 h with IBMX in the presence or absence of 1 mM N^G-monomethyl-L-arginine (LNMA, Alexis), added at the same time as IBMX [20]. cGMP levels were determined in cell lysate and conditioned medium using a specific radioimmunoassay (Immunotech). Rat aortic smooth muscle cells (kind gift of Prof. U. Ruegg, Pharmacy School, Lausanne), used as positive control, were prepared from aortae of male Wistar Kyoto rats (200–300 g) and were cultured in DMEM supplemented with essential and non-essential amino acids, vitamins, 10 μg/ml ciproxin, and 10% fetal calf serum [30]. Rat aortic smooth muscle cells were used between passages 6 and 11.

2.5. Plasmid construction and transfection

Human iNOS coding cDNA [31] was subcloned into the *NotI* site of the pCIpuro vector which contains a puromycin resistance gene (kindly provided by Dr. J. Mirkovitch, Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland). HEK cells were transiently transfected with either purohiNOS or control vector using DMRIE-C reagent (Invitrogen) according to manufacturer's instruction. iNOS activity was assessed indirectly 72 h after the transfection by measurement of nitrite accumulation during 48 h in the conditioned medium as previously described [25].

2.6. Immunofluorescence

Transfected HEK cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 in PBS for 2 min. Immunostaining was carried out by incubation with NO53 anti-iNOS antibody (kind gift of Dr. Mumford, Merck Research Laboratories, Rahway, NJ, USA) at a dilution of 1:10,000 followed by a revelation using Cy3-conjugated anti-rabbit-immunoglobulin (Ig) G antibodies (Jackson Immunoresearch Laboratories) at a dilution of 1:200 for 45 min. Immunofluorescence was observed with a Zeiss Axiophot immunofluorescence microscope.

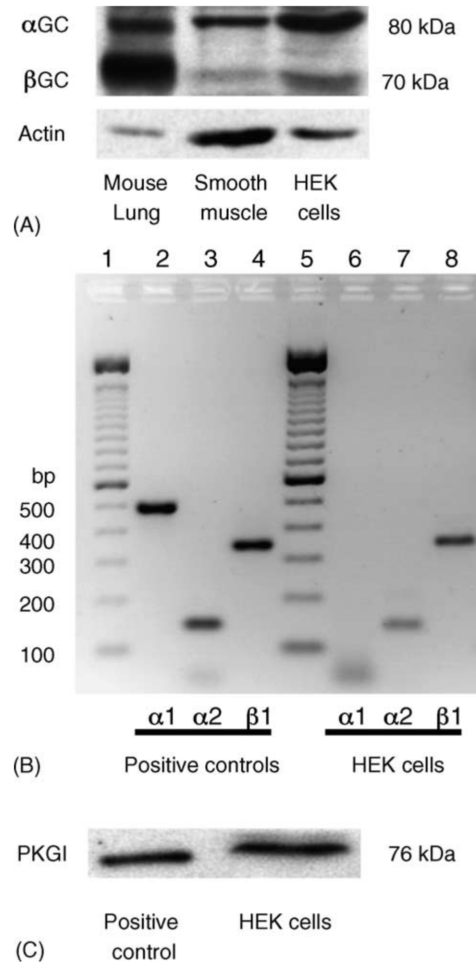

Fig. 1. Presence of NO–cGMP signaling pathway components in HEK cells. (A) Western blot analysis of α and β NO-sensitive GC subunits expression. Homogenate of mouse lung and human smooth muscle extracts were used as positive control. Representative of four independent experiments. (B) RT–PCR of α and β NO-sensitive GC subunits: 100 bp DNA ladder marker (lanes 1 and 5), α1 cDNA (lane 2), HL-60 cells cDNA (lane 3), β1 cDNA (lane 4) used as positive control. HEK cells cDNA: no α1 fragment at 488 bp is detected (lane 6), α2 fragment at 145 bp (lane 7), β1 fragment at 345 bp (lane 8). Representative of two independent experiments. (C) Western blot analysis of PKGI expression. Human umbilical vein endothelial cells transfected with PKGI cDNA were used as positive control. Representative of six independent experiments.

3. Results

3.1. Presence of NO–cGMP signaling pathway components in HEK cells

The presence of the various components of the NO–cGMP signaling pathway was verified in HEK cells. To be functional and active, NO-sensitive GC has to be present in heterodimeric form composed by α and β subunits. Consistent with other studies [19,23], these two subunits were present in HEK cells as shown by Western blot (Fig. 1A). The antibodies recognized a major band at 80 kDa and a minor band at 70 kDa, corresponding to α and β NO-sensitive GC subunits, respectively, in HEK cells and human smooth muscle cells. To further characterize the

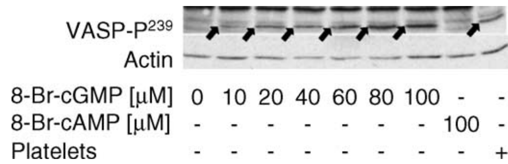

Fig. 2. Effect of exogenous cGMP on VASP phosphorylation. Western blot analysis of VASP-Ser^{239} phosphorylation (VASP-P^{239}) on extracts from HEK cells treated during 30 min with 10–100 μM 8-Br-cGMP or 100 μM 8-Br-cAMP. Human platelets were used as positive control. Representative of five independent experiments.

expression of NO-sensitive GC in HEK cells, RT–PCR was performed and allowed to determine that α2 and β1 NO-sensitive GC subunits were expressed in HEK cells (Fig. 1B). Finally, the presence of cGMP target PKGI was demonstrated by Western blot analysis (Fig. 1C). Taken together these results confirm and characterize previous data showing the existence of cGMP–PKGI pathway in HEK cells [9,32,33].

3.2. Effect of exogenous cGMP on VASP phosphorylation

In order to explore the cGMP–PKGI pathway function in HEK cells, the latter were exposed to 8-Br-cGMP, a stable lipophilic analog of cGMP, and phosphorylation of VASP on serine 239, which is a preferential site of PKGI phosphorylation [15,18,34–38], was investigated. A dose–response curve of 8-Br-cGMP (Fig. 2) showed that VASP-Ser^{239} phosphorylation was already detectable with 10 μM 8-Br-cGMP while 100 μM of 8-Br-cAMP had no effect, buttressing the absence of PKA and PKG cross activation at these cyclic nucleotides concentrations in HEK cells [19]. These results confirm the specificity of VASP-Ser^{239} phosphorylation site for cGMP effect and the efficiency of the exogenous cGMP to phosphorylate VASP on HEK cells.

3.3. Stimulation of NO-sensitive GC-PKGI pathway by NO donors

In order to verify if VASP-Ser^{239} phosphorylation in HEK cells might be the consequence of cGMP production by NO-sensitive GC, cells were treated with different NO donors and ODQ, a specific inhibitor of NO-stimulated activity of NO-sensitive GC [39]. First, the production of cGMP following exposure to NO donors DEA/NO and DETA/NO, with half-life of 2 min and 20 h (at pH 7.4, 37 °C), respectively, was determined. In HEK cells treated during 10 min with 10 μM DEA/NO, the production of cGMP was of 4.6 ± 1.2 pmol cGMP/min/mg protein and was significantly higher compared to non treated HEK cells (Mann–Whitney test, $P < 0.01$, $n = 5$–6, Fig. 3A). Treatment of HEK cells during 10 min with 10 μM ODQ had no effect on basal production of cGMP by these cells (Fig. 3A). However, DEA/NO-dependent production of cGMP was prevented by treatment with 10 μM OQD

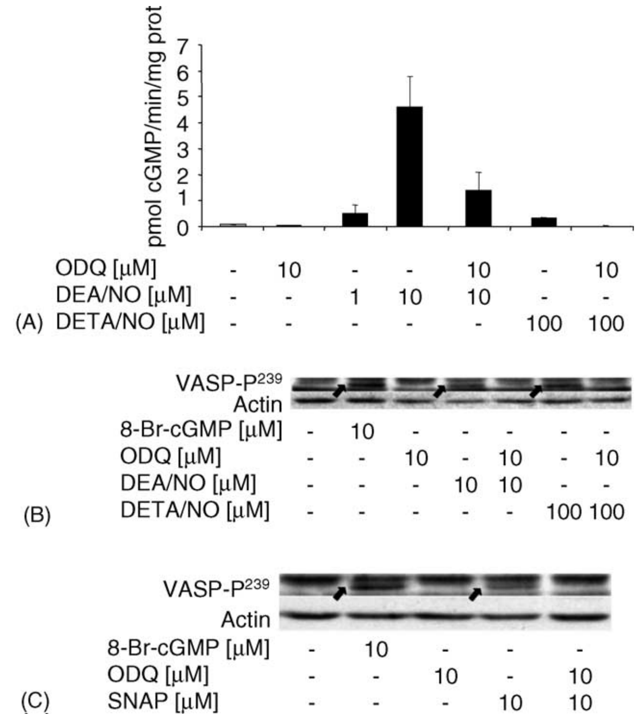

Fig. 3. Stimulation of the NO-sensitive GC-PKGI pathway by NO donors. (A) Measurement of cGMP levels in HEK cells treated during 10 min with 10 μM DEA/NO or 100 μM DETA/NO in the presence or absence of 30 min pretreatment with 10 μM ODQ ($n = 3$–6). (B) Western blot analysis of VASP-P^{239} on extracts from HEK cells treated during 30 min with 10 μM 8-Br-cGMP or DEA/NO, or during 60 min with 100 μM DETA/NO, in the presence or absence of 30 min pretreatment with 10 μM ODQ. (C) Western blot analysis of VASP-P^{239} on extracts from HEK cells treated during 10 min with 10 μM 8-Br-cGMP or SNAP, in the presence or absence of 30 min pretreatment with 10 μM ODQ. All blots are representative of three independent experiments.

during 10 min (Mann–Whitney test, $P < 0.05$, $n = 3$–6, Fig. 3A). Same results were obtained after 10 min exposure to 100 μM DETA/NO which significantly increased cGMP production to 0.30 ± 0.09 pmol cGMP/min/mg protein (Mann–Whitney test, $P < 0.01$, $n = 3$–5, Fig. 3A), while ODQ treatment prevented this production (Mann–Whitney test, $P < 0.05$, $n = 3$, Fig. 3A). As positive control, cultured rat aortic smooth muscle cells were stimulated during 10 min with 10 μM DEA/NO, which led to the production of 10.6 ± 2.0 pmol cGMP/min/mg protein ($n = 3$).

To investigate whether NO donors-induced cGMP production was able to stimulate PKGI, VASP-Ser^{239} phosphorylation was analyzed. Both DEA/NO and DETA/NO were able to induce VASP phosphorylation on serine 239 (Fig. 3B). Furthermore, NO-donors-induced VASP-Ser^{239} phosphorylation was prevented by ODQ, confirming the implication of NO-sensitive GC (Fig. 3B).

Same results were obtained with the NO donor SNAP, which is a nitrosothiol with a half-life of 5.5 h (at pH 7.4, 37 °C). HEK cells treated with 10 μM SNAP during 10 min showed a production of 2.8 ± 0.8 pmol cGMP/min/mg protein (Mann–Whitney test, $P < 0.005$ versus control, $n = 3$–5). Accordingly, SNAP was able to induce

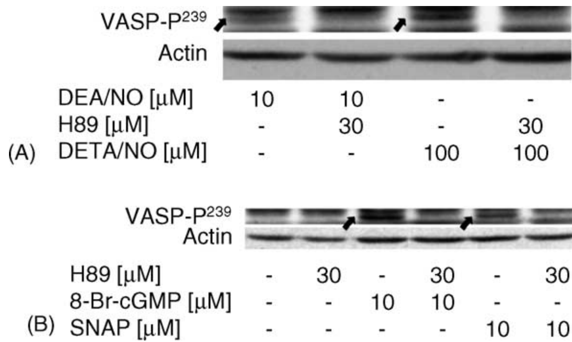

Fig. 4. Effect of kinase inhibitor H89 on VASP-P^{239}. (A) Western blot analysis of VASP-P^{239} on extracts from HEK cells treated during 30 min with 10 μM 8-Br-cGMP or DEA/NO, or during 60 min with 100 μM DETA/NO in the presence or absence of 30 min pretreatment with 30 μM of H89. (B) Western blot analysis of VASP-P^{239} on extracts from HEK cells treated during 30 min with 10 μM 8-Br-cGMP or SNAP in the presence or absence of 30 min pretreatment with 30 μM H89. Representative of three independent experiments.

VASP-Ser^{239} phosphorylation, which was prevented by ODQ treatment (Fig. 3C).

In order to determine the implication of PKGI on VASP-Ser^{239} phosphorylation, the cyclic nucleotides-dependent protein kinase inhibitor H89 was used. Indeed, the in vitro more specific PKG inhibitor KT5823 had, as described by others using this compound in intact cells [40,41], a weak effect compared to H89 (data not shown). However, since in HEK cells up to 100 μM 8-Br-cAMP did not induce VASP-Ser^{239} phosphorylation (Fig. 2), it is reasonable to assume that in our experimental conditions H89 is not acting on a PKA-dependent phosphorylation. A pre-treatment during 30 min with 30 μM H89 was able to inhibit VASP-Ser^{239} phosphorylation in HEK cells treated with either 10 μM DEA/NO or 100 μM DETA/NO (Fig. 4A). The same inhibition by H89 was observed after cell exposure to 10 μM 8-Br-cGMP or 10 μM SNAP (Fig. 4B). These results suggest the implication of PKGI in VASP phosphorylation by NO donors in HEK cells.

3.4. Effect of endogenous production of NO

VASP phosphorylation by NO has been mostly investigated downstream of constitutive NOS pathway although several examples of iNOS-dependent increase of cGMP have been described [20,21]. To address the question whether iNOS is able to induce VASP phosphorylation, HEK cells were transiently transfected with iNOS cDNA. Immunostaining revealed that iNOS protein was expressed only in purohiNOS, but not in mock transfected HEK cells (Fig. 5A). As a consequence, purohiNOS transfected cells produced 107 ± 13 pmol NO_2^-/min/mg protein, which was significantly higher compared to mock transfected cells (t-test, $P < 0.001$, n = 4–9, Fig. 5B). Addition of LNMA, an inhibitor of iNOS activity, suppressed 88% of nitrite accumulation (data not shown), confirming the implication of iNOS in nitrite production. While in mock transfected cells cGMP accumulation was not different from control untransfected cells (data not shown), expression of iNOS resulted in 9.6 ± 1.1 pmol cGMP/10^5 cells after 6 h (n = 3). Addition of 1 mM LNMA during the same period led to 71% inhibition of cGMP accumulation (paired t-test, $P < 0.05$, n = 3), confirming a role of iNOS in cGMP accumulation. Finally, iNOS-dependent VASP phosphorylation on serine 239 could be also detected (Fig. 5C).

Taken together these results demonstrate that iNOS is able to increase cGMP leading to VASP phosphorylation in HEK cells.

4. Discussion

The NO–cGMP pathway is a major component of vital functions in cardiovascular system, intestine, kidney, bone, central nervous system and reproductive system [4]. In this study, we have first defined that α2/β1 NO-sensitive GC is expressed in HEK cells. Second, NO–cGMP–PKGI–VASP phosphorylation pathway was characterized, extending previous studies using the same cells, which showed cGMP production upon NO donors exposure [19,23] or VASP phosphorylation following cGMP administration [32]. Finally, we have demonstrated that expression of iNOS leads to production of cGMP and VASP phosphorylation.

Levels of cGMP production upon exposure to 10 μM DEA/NO are within the range of cGMP production previously observed in HEK cells using the same NO-donor [23] or 100 μM sodium nitroprussiate [19]. Although the amount of cGMP is low compared to NO-sensitive GC transfected cells [42], it was within the range of cGMP levels obtained in DEA/NO stimulated cultured smooth muscle cells. We have determined in this study that NO-sensitive GC expressed in HEK cells is constituted by α2/β1 heterodimer. C-terminal peptide of α2 subunit interacts with a PDZ domain of the postsynaptic scaffold protein PSD-95 suggesting that the α2/β1 heterodimer can be recruited to the membrane [43]. Recent data indicate that NO-sensitive GC compartmentation may be involved in efficient delivery of NO, which can otherwise be scavenged by other heme-containing protein (e.g. myoglobin), at least in heart [44]. Specific subcellular distribution may also be associated to targeted cGMP delivery. Indeed, cGMP target PKGI, although mostly cytosolic, has been also found associated to membrane ruffles in A549 epithelial cells [32,45]. Although α2 NO-sensitive GC protein is mostly abundantly expressed in brain, it was also detected in lung, aorta, colon, heart, liver and kidney in adult mice [46], therefore it is not surprising to observe its expression in a cell line established from human embryonic kidney. Nothing is known about NO-sensitive GC expression in embryogenesis in mammals but in medaka fish α2 subunit is the earliest GC α subunit expressed during embryogenesis, being in parallel with PGKI expression [47]. Injection of medaka fish embryo with morpholino antisense oligo-

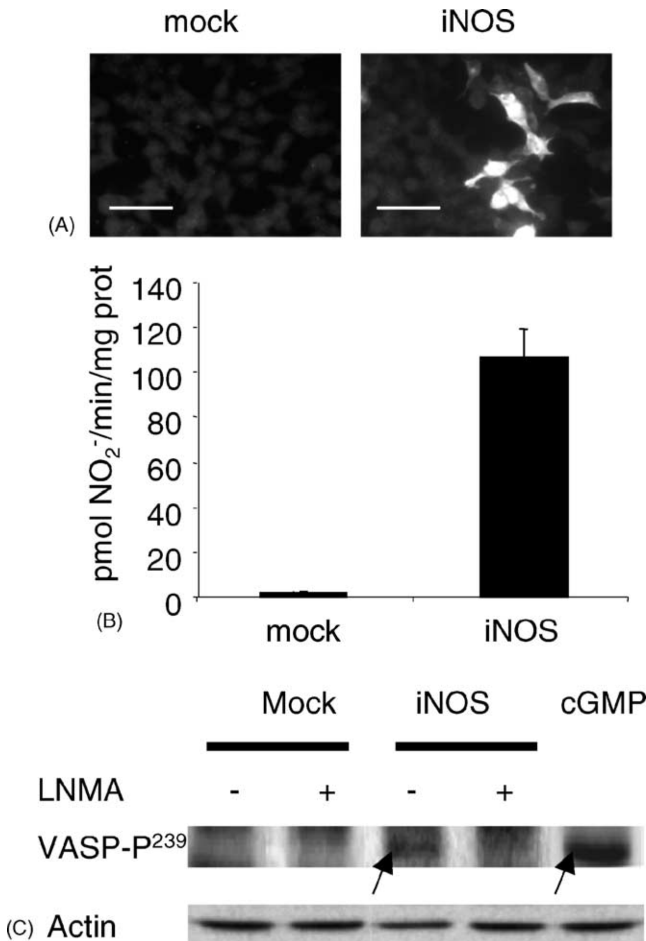

Fig. 5. Effect of endogenous production of NO on VASP-P^{239}. (A) Immunostaining of HEK cells transiently transfected with mock DNA or iNOS cDNA. (B) Measurement of nitrite accumulation in HEK cells transiently transfected with mock DNA or iNOS cDNA ($n = 4$–9). (C) Western blot analysis of VASP-P^{239} on extracts from HEK cells transiently transfected with mock DNA or iNOS cDNA, treated or not with 1 mM LNMA during 48 h. As positive control the extract from HEK cells treated during 30 min with 10 μM 8-Br-cGMP was used. Representative of three independent experiments.

nucleotide directed against NO-sensitive GC leads to several abnormalities indicating a critical role of NO–cGMP pathway in medaka fish embryogenesis [47].

Consistent with studies mentioned above and previous data using the same cells [19,32,42], PGKI was found expressed in HEK cells and its activity following stimulation by cGMP was determined by phosphorylation of VASP on serine 239, which is a widely used strategy to assess PGKI activity [18,32]. This tool allowed us to observe PGKI activity after exogenous cGMP administration, stimulation of endogenous NO-sensitive GC using NO donors or endogenous iNOS-derived NO. Although VASP was first found as a major phosphorylated protein in platelets following stimulation with NO donors [16], this is the first time that VASP phosphorylation is associated to iNOS activity. As shown previously in another cell line [20], iNOS induced cGMP accumulation that was prevented by addition of NOS inhibitor LNMA. The latter also inhibited iNOS-induced phosphorylation of VASP confirming the role of iNOS in VASP phosphorylation. A direct comparison to efficiency of NO-donors or administration of cGMP is not possible since we estimated that only about 5% of cells were expressing iNOS.

The biological implication of iNOS-induced phosphorylation of VASP can be inferred from known functions of VASP. The latter increases F-actin formation in vitro [48–50] and regulates actin polymerization in vivo [50–53]. Phosphorylation of VASP inhibits F-actin binding and actin polymerization in vitro [49,54]. In addition, cGMP mediated phosphorylation of VASP inhibits serum-response element-dependent transcription indicating that NO–cGMP–PGKI pathway affects not only cell shape and migration but also gene expression [55]. Most of our investigations were performed on short-term exposure to NO donors or cGMP and neither cytoskeletal changes or gene expression were investigated. However, these processes were potentially ongoing after transfection with iNOS cDNA, where we showed that expression of iNOS leads to production of cGMP and phosphorylation of VASP. Future investigations should explore this issue which may be involved also in downregulating the NO–cGMP signaling pathway.

This study adds a new example to the growing list of data showing a link between iNOS and cGMP signaling [20,21,56–58]. Although our observations were obtained in cultured cells, an autocrine and iNOS-dependent signaling may have in vivo relevance in cells where all signaling components are expressed. Despite an abundant research on nitrosative stress, which is mostly related to pathology, it is possible that iNOS expression is involved in physiological response through cGMP signaling.

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