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Dual actions of dephostatin on the nitric oxide/cGMP-signalling pathway in porcine iliac arteries

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Abstract

We examined the effects of the nitrosoamine dephostatin on the nitric oxide (NO)/cyclic guanosine 3′,5′-monophosphate (cGMP)-signalling in porcine iliac arteries. Dephostatin has been characterised as a tyrosine phosphatase inhibitor, but Western blot analyses showed that dephostatin did not augment tyrosine phosphorylation of arterial proteins. However, dephostatin relaxed pre-contracted arteries, and this effect was antagonised by the soluble guanylyl cyclase inhibitor 1*H*[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ). Furthermore, dephostatin increased the cGMP content and the serine phosphorylation of vasodilator-stimulated phosphoprotein. Dephostatin also inhibited the relaxation induced by acetylcholine and the NO-donor *S*-nitroso-*N*-acetyl-penicillamine (SNAP). In contrast, dephostatin did not affect the NO-dependent actions of 1,2,3,4-Oxatriazolium, 3-(3-chloro-2-metylphenyl)-5-[[(4methylphenyl)sulfonyl]amino]-hydroxide inner salt (GEA 3175). Measurement of NO revealed that dephostatin accelerated the consumption of NO. In conclusion, dephostatin exerts dual effects on the NO/cGMP-signalling pathway in iliac arteries. The drug actions included scavenging of NO, but also stimulation of cGMP production. These effects were not related to inhibition of tyrosine phosphatases.

Keywords: Dephostatin; cGMP; GEA 3175; Nitric oxide; Protein phosphorylation; Relaxation; SNAP

1. Introduction

Smooth muscle contraction and relaxation are tightly balanced processes, regulated by changes in cytosolic free Ca^{2+} concentration and cyclic nucleotide levels (Schlossmann et al., 2003; Somlyo and Somlyo, 2003). For instance, nitric oxide (NO) activates soluble guanylyl cyclase leading to synthesis of intracellular cyclic guanosine 3′,5′ monophosphate (cGMP). The increase in cGMP results in inhibition of cytosolic Ca^{2+} rises and subsequent relaxation of vascular smooth muscle tissue (Carvajal et al., 2000). Increased tyrosine phosphorylation of smooth muscle proteins has also been shown to influence on smooth muscle contraction (Hughes and Wijetunge, 1998). The degree of tyrosine phosphorylation depends on the interplay between protein tyrosine kinases and protein tyrosine phosphatases. It has been shown that the protein tyrosine phosphatase inhibitor orthovanadate, increased tyrosine phosphorylation and, on that account, induced vasoconstriction (Srivastava and St-Louis, 1997). Consequently, drugs that interact with protein tyrosine phosphatases may significantly influence on vascular smooth muscle tone.

The nitrosoamine dephostatin, originally isolated from a streptomyces strain, has been characterised as a non-selective, membrane permeable and competitive inhibitor of protein tyrosine phosphatases (Imoto et al., 1993). Previous investigations have shown that dephostatin affects a broad range of molecular and functional responses in smooth muscle cells. For example, the nitrosoamine increases voltage-operated Ca^{2+}-channel current in rabbit ear artery smooth muscle cells (Wijetunge et al., 1998) and inhibits adenosine A_{2a}-receptor mediated inhibition on Ca^{2+} current in rat cerebral smooth muscle cells (Murphy et al., 2003). These effects were preferentially due to increased phosphorylation of proteins on tyrosine residues. Dephostatin has also been found to increase cytosolic free Ca^{2+} concentration in isolated rat pancreatic acinar cells (Lajas et al., 2000). This effect of dephostatin involved an oxidising action, but was unrelated to inhibition of protein

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tyrosine phosphatases. Taken together, several published papers point to the interplay between dephostatin and cytosolic Ca^{2+} signalling. On the other hand, the possible interaction between dephostatin and cyclic nucleotides is presently unknown.

This study was designed to investigate the effects of dephostatin on the NO/cGMP-signalling pathway in porcine iliac arteries. Particular emphasis was placed on the mechanisms of drug action and to clarify possible phosphatase-independent effects of dephostatin. The influence of dephostatin on *S*-nitroso-*N*-acetyl-penicillamine (SNAP)- and 1,2,3,4-Oxatriazolium, 3-(3-chloro-2-metylphenyl)-5-[[(4methylphenyl)sulfonyl]amino] hydroxide inner salt (GEA 3175)-induced smooth muscle relaxation was evaluated. These two NO-containing compounds have different NO-liberating and cGMP-elevating properties (Asplund Persson et al., 2004). Furthermore, the effect of dephostatin on acetylcholine-induced, endothelium-dependent relaxation was evaluated. The results revealed that dephostatin interfered directly with NO molecules, and reduced NO-induced relaxation, cGMP accumulation, and serine-specific phosphorylation of vasodilator-stimulated phosphoprotein (VASP). Interestingly, these actions were not due to inhibition of protein tyrosine phosphatases.

2. Materials and methods

2.1. Tissue preparations and tension studies

Porcine iliac arteries were obtained from a local slaughterhouse within 30 min of slaughter. Specimens were immersed in Krebs bicarbonate buffer (Na^+ 137 mM, Cl^- 134 mM, K^+ 5.9 mM, Ca^{2+} 2.5 mM, Mg^{2+} 1.2 mM, HCO_3^- 15.4 mM, $H_2PO_4^-$ 1.2 mM and glucose 5.6 mM, pH 7.4). The Krebs buffer was equilibrated with 5% CO_2 and 95% O_2 at 37 °C. The arteries were dissected free from surrounding tissues, cut into rings of 3–5 mm and then cut open forming arterial strips. In some arterial strips the endothelial layer was removed by gently rubbing the luminal side with a cotton stick. The arterial strips were mounted under a resting tension of 20 mN in organ baths containing Krebs buffer and were allowed to equilibrate for about 30–60 min. Before initiation of experiments passive tension was 5–15 mN. The arterial strips were repeatedly contracted with the α_1-adrenoceptor agonist phenylephrine (1 μM) or the thromboxane A_2 analogue U46619 (0.01 μM). Between each addition of phenylephrine or U46619, the arterial preparations were thoroughly washed for 30 to 50 min with Krebs buffer which made tension return to basal level. The functionality of the endothelium was examined by adding acetylcholine. Arterial preparations that responded to acetylcholine (1 μM) with less than 50% relaxation were excluded. Changes in muscle tension were measured isometrically using Grass FT .03 isometric gauge transducers and Grass Polygraphs (Grass Instruments Inc., Quincy, MA, USA).

In order to study dephostatin-induced relaxation, iliac arteries were contracted with phenylephrine. After approximately 15 min, when a stable contraction was achieved, dephostatin (up to a concentration of 10 μM) was added. The soluble guanylyl cyclase inhibitor 1*H*[1, 2, 4]oxadiazolo[4, 3-*a*]quinoxalin-1-one (ODQ; 50 μM), was used to evaluate the mechanism behind the dephostatin-induced relaxation. In these experiments, ODQ was present for 15 min prior addition of dephostatin. In another experimental design, dephostatin was added 15 min before phenylephrine stimulation. Dephostatin was dissolved in dimethyl sulfoxide. For that reason, arterial preparations exposed to the solvent alone (0.2% dimethyl sulfoxide) was analysed in parallel.

In other series of experiments, the effect of dephostatin on acetylcholine- and NO-donor-induced relaxation was examined. Iliac arteries were contracted with phenylephrine or U46619. After 15 min, when a stable contraction was achieved, the solvent was added and after additional 15 min SNAP (1 μM), GEA 3175 (0.1 μM), or acetylcholine (0.01–0.1 μM) was introduced. The NO-mediated relaxation was registered for 20–30 min. Thereafter, the preparations were thoroughly washed and re-exposed to phenylephrine. When a stable contraction was reached the muscle preparations were exposed to either dimethyl sulfoxide or dephostatin (10 μM) for 15 min and then SNAP, GEA 3175 or acetylcholine were added. The effect of longer incubation times (30 or 60 min) with dephostatin (10 μM) was also investigated.

Dephostatin, SNAP, GEA 3175, and acetylcholine-induced relaxation was calculated as % of phenylephrine-induced contraction.

2.2. Determination of the cGMP content

Porcine iliac arteries were cut into rings and put into plastic vials containing oxygenated Krebs buffer at 37 °C for 30–60 min. After 10 min of incubation in new vials with Krebs buffer supplemented with the phosphodiesterase inhibitor 3-isobutyl-L-methyl-xanthine (IBMX; 100 μM), the arterial rings were treated with dephostatin (10 μM) for 15 min before addition of SNAP (1 μM) or GEA 3175 (0.1 μM). After additional 2 min, specimens were immediately frozen by immersing them in acetone and solid CO_2. Thereafter, the arterial preparations were homogenised in 3 ml ice-cold trichloroacetic acid (10%) in a glass–glass homogeniser. The homogenates were then centrifuged at 4000 ×*g* for 15 min in 4 °C and the supernatants were extracted with 4×3 ml of water-saturated diethyl ether. The aqueous phase was frozen to dryness in a vacuum freezer for 18 h and then reconstituted in Na–acetate buffer (50 mM, pH 6.2). The levels of cGMP were determined by radioimmunoassay (Axelsson et al., 1988) and all determinations were performed in duplicate. The amount of cGMP was related to the protein content in the vessel preparations. The remaining pellet was boiled in NaOH (1 mM). Protein-determinations were done spectrophotometrically (SPECTRA MAX 340, Molecular Devices, Sunnyvale, CA, USA), by measuring absorbance at 595 nm using the Coomassie Blue protein detection reagent (Pierce, Rockford, IL, USA).

2.3. Analysis of protein phosphorylation

Arterial rings were exposed to dephostatin, SNAP, GEA 3175 alone, and dephostatin combined with SNAP or GEA

3175 as described in Section 2.2 *Determination of the cGMP content*. The specimens were homogenised in liquid nitrogen and the pulverised tissue was mixed with ice-cold lysis buffer (NaCl 150 mM, Tris 20 mM, EDTA 5 mM, Na_3VO_4 2 mM, leupeptin 4 μg/ml and phenylmethylsulfonyl fluoride 60 μg/ml). This mixture was then frozen and thawed on ice 3 times and finally centrifuged twice at 5000 ×*g* for 10 min at 4 °C. The protein content in the supernatant was measured with the Coomassie Blue reagent. Each sample from one animal was diluted to the same protein-concentration. The supernatant was mixed with Laemmli buffer (Bio-Rad, Hercules, CA, USA) composed of 62.5 mM Tris–HCl, 25% glycerol, 2% sodium dodecyl sulphate (SDS), 0.01% bromophenol blue, and mercaptoethanol 5%, pH 6.8) 1 : 2 (vol : vol) and heated at 95–97 °C for 5 min. The samples were stored at −70 °C until used. The heating procedure was performed again before separating the aliquots on gradient 4–20% (tyrosine phosphorylation) or 7.5% (VASP-phosphorylation) SDS-polyacrylamide gels (SDS-PAGE) (Bio-Rad) using a Mini-PROTEAN II Electrophoresis Cell (Bio-Rad). The proteins were transferred to polyvinylidene difluoride membranes (Millipore Corporation, Billerca, MA, USA) using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). The membranes were blocked for 1 h at room temperature or overnight at 4 °C with 5% (*w*/*v*) dry milk and 0.2% (*v*/*v*) Tween 20 in PBS pH 7,4 composed of phosphate-buffer 10 mM, and NaCl 150 mM to minimise unspecific binding. A mouse monoclonal antibody (PY-99) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) directed towards phosphotyrosine was used at a dilution of 1 : 8000 and a mouse monoclonal antibody (16C2) (Alexis Biochemicals, Qbiogenic, Inc., CA., USA) directed towards serine^{239} phosphorylated VASP was used at a dilution of 1 : 1000. The secondary antibody was a horseradish peroxidase conjugated goat anti-mouse (Santa Cruz Biotechnology) and diluted to 1 : 10,000. The antibodies were diluted into 0.2% (*v*/*v*) Tween 20 in PBS and the membranes were rinsed between incubations in 0.2% (*v*/*v*) Tween 20 in PBS. The membranes were detected using enhanced chemiluminescence Western blotting detection reagents (Amersham Biosciences, UK) in LAS-1000 imaging Analyser (Fuji Photo Film, Tokyo, Japan).

2.4. Measurement of NO

Amperometric measurements of NO were performed using the ISO-NO Mark II NO-meter, (World Precision Instruments Inc., Sarasota, USA). The analogue signals from the instrument was digitalized using a two-channel data-acquisition system connected to a Pentium II PC. The analyses were performed in HEPES buffer (pH 7.4) composed of NaCl 145 mM, KCl 5 mM, $MgSO_4$ 1 mM, $CaCl_2$ 1 mM, glucose 10 mM, and HEPES 10 mM, under constant stirring (800 rpm) and at 37 °C. The release of NO from SNAP (10 μM) was analysed in the absence and presence of dephostatin (10 or 30 μM). Measurements were done in the same manner with NO gas bubbled in 5 ml distilled water for 5 min. Thereafter, 2 μl of the NO-containing solution was added to 2 ml of HEPES buffer. The liberation of NO from dephostatin was also investigated in the presence of arterial preparations. The vessel strips were prepared as described in Section 2.1 *"Tissue preparations and tension studies"*.

2.5. Statistical analysis

Results are expressed as the mean values ± standard error of the mean (S.E.M.). Statistical significance was tested with one-way analysis of variance (ANOVA), with Newman–Keuls as post hoc test for multiple comparisons or Students *t*-test for comparisons between two groups. Data were analysed using GraphPad Prism™ v. 4 (GraphPad Software, San Diego, CA, USA).

2.6. Drugs

SNAP, dephostatin and ODQ were from Alexis Co. (San Diego, CA, USA). GEA 3175 was a generous gift from GEA Pharmaceutical (Copenhagen, Denmark). SNAP, dephostatin and GEA 3175 were dissolved in dimethyl sulfoxide; the final concentration of dimethyl sulfoxide in the different experimental setups did not exceed 0.2%. These drugs are sensitive to light and therefore the solutions were kept in foiled-wrapped vials. Phenylephrine, U46619 (9,11-Dideoxy-11α, 9α-epoxy-methano-prostaglandin F2α), acetylcholine and 3-isobutyl-L-methylxanthine were from SIGMA (St. Louis, MO, USA).

3. Results

3.1. Effect of dephostatin on NO-induced relaxation of iliac arteries

In the first experimental design, the effect of dephostatin on vascular smooth muscle tone was clarified. The arterial strips were stimulated with phenylephrine (1 μM) and then exposed to dephostatin up to a concentration of 10 μM. As shown in Fig. 1, dephostatin (10 μM) caused a slow relaxation of precontracted porcine iliac arteries. The results revealed that the response of the arterial preparations to dephostatin was variable (see original traces in Figs. 1 and 3 for comparison). More specifically, in 24 of 35 blood vessel preparations, dephostatin produced a slow vasorelaxation. The magnitude of relaxation (15 min after the addition of dephostatin) was 27.3 ± 3.9% (mean value ± S.E.M., $n = 24$) of phenylephrine-induced contraction. Lower doses of

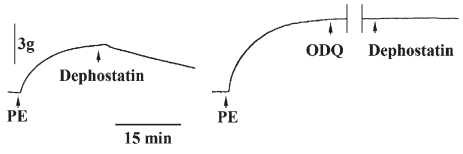

Fig. 1. The effect of dephostatin on precontracted iliac arteries. Preparations of porcine iliac arteries were contracted with phenylephrine (PE; 1 μM). When stable contractile response was obtained, the vessel preparations were exposed to the nitrosoamine dephostatin (10 μM; the original trace to the left). In some experiments, precontracted arteries were incubated with the soluble guanylyl cyclase inhibitor ODQ (50 μM), and after additional 15 min exposed to dephostatin (the original trace to the right). The figure shows one representative recording from totally 3 experiments.

dephostatin did not affect the precontracted vessels (data not shown). As shown in Fig. 1, pretreatment for 15 min with the soluble guanylyl cyclase inhibitor ODQ (50 μM) abolished dephostatin-induced relaxations ($n=3$). The influence of dephostatin on U46619 (0.01 μM)-stimulated arteries was also investigated. In common with results obtained by using phenylephrine, dephostatin elicited vasorelaxation in 7 of 9 U46619-contracted vessel preparations. The degree of dephostain-induced relaxation (15 min after the addition of the compound) was 26.9±5.4% (mean value±S.E.M., $n=7$) of U46619-induced contraction. In another experimental design, the vessels were pretreated with dephostatin for 15 min and then exposed to phenylephrine. Low doses (0.01 to 1 μM) of dephostatin did not affect the contractile response, but treatment with 10 μM of dephostatin significantly inhibited a subsequent phenylephrine-induced vasocontraction (Fig. 2).

In another series of experiments, phenylephrine-precontracted iliac arteries were exposed to acetylcholine or to the NO-containing compounds SNAP and GEA 3175. As shown in Fig. 3, both the *S*-nitrosothiol SNAP, the oxatriazole derivative GEA 3175, and acetylcholine induced relaxation of the arterial preparations. The relaxations were found to be highly reproducible. For instance, acetylcholine (0.1 μM)-mediated relaxations were 81.4±3.6%, 72.4±4.8%, and 77.0±3.9% after, respectively, the first, second and third phenylephrine-induced contraction, mean±S.E.M, $n=9$. Similar results were obtained by using SNAP (1 μM) and GEA 3175 (0.1 μM) (data not shown). Notably, higher doses of GEA 3175 (1 μM) produced nearly 100% relaxation, but the effect of the drug was difficult to washout. Consequently, GEA 3175 was used in a 10-fold lower concentration than SNAP. The results revealed that the SNAP-induced relaxation was markedly reduced in dephostatin (10 μM; 15 min pre-incubation time)-treated blood vessels (original traces shown in Fig. 3 and results summarised in Fig. 4A). On the contrary, dephostatin did not affect acetylcholine- or GEA 3175-induced relaxation (Figs. 3, 4B and F). However, longer incubation time with dephostatin (60 min), resulted in a significant inhibition of acetylcholine-induced relaxation of iliac arteries (Fig. 4G and H). On the other hand, exposure of the tissue to dephostatin for 60 min did not affect subsequent GEA

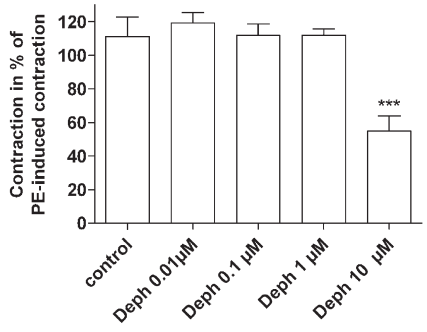

Fig. 2. The effect of dephostatin on phenylephrine-induced contraction. Arterial strips was pretreated for 15 min with dephostatin (Deph; 0.01–10 μM) and then exposed to phenylephrine (PE; 1 μM). Data are presented as mean±S.E.M., $n=4–6$. Statistical significance was tested with ANOVA, followed by Newman–Keuls multiple comparison test as post hoc test (***$P<0.001$).

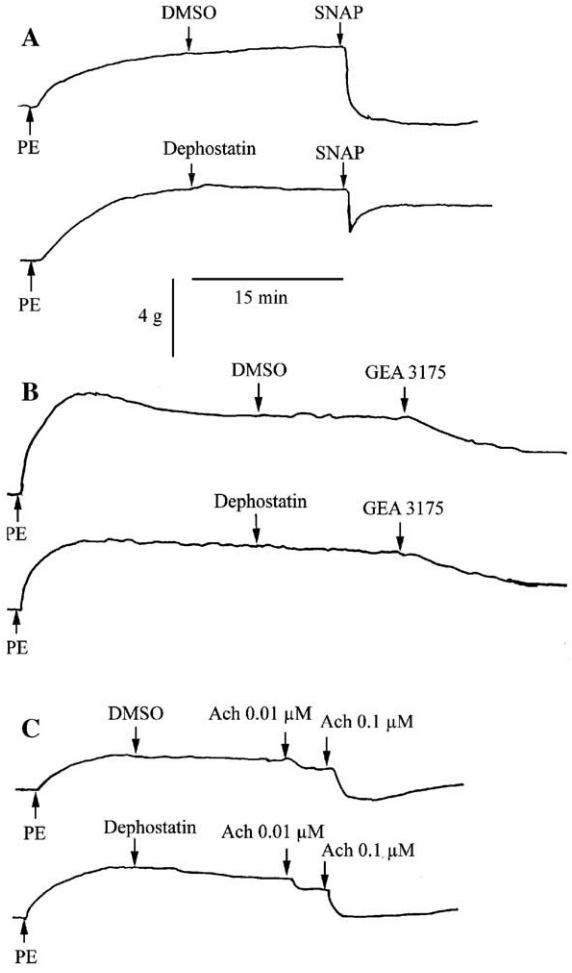

Fig. 3. Recordings illustrating the effect of dephostatin on SNAP, GEA 3175 and acetylcholine-induced relaxation. Iliac arteries were precontracted with phenylephrine (PE; 1 μM) and, when a stable contraction was achieved, the NO-containing compounds SNAP (1 μM; A), GEA 3175 (0.1 μM; B) or acetylcholine (Ach; 0.01–0.1 μM; C) was introduced. The preparations were thoroughly washed and once again exposed to phenylephrine. When a stable contraction was reached the muscle preparations were exposed to dephostatin (10 μM) or the solvent dimethyl sulfoxide (DMSO) for 15 min and then SNAP, GEA 3175 or acetylcholine was added, as indicated by the arrows. The figure shows one of at least 6 different recordings.

3175-mediated relaxation (data not shown). We also found that dephostatin reduced SNAP-mediated relaxation in U46619-precontracted arterial strips (Fig. 4C). The potential role of endothelial cells was evaluated by comparing the effect of dephostatin on vessel preparations with intact or denuded endothelium. The results revealed that the antagonising action of dephostatin on SNAP-induced relaxation was equally pronounced in the absence of the endothelium (Fig. 4D). It is well known that NO and NO-releasing agents produce a more or less rapid desensitisation. The nitrosoamine dephostatin contains a NO-moiety, and theoretically, the compound could have desensitised the vessels to subsequent addition of SNAP. To exclude or confirm the involvement of a desensitisation phenomenon, the arterial strips were pre-treated with a low concentration (0.01 μM) of SNAP for 15 min followed by a higher dose (1 μM) of SNAP. The results showed that the arterial preparations relaxed in a normal manner to the second, higher

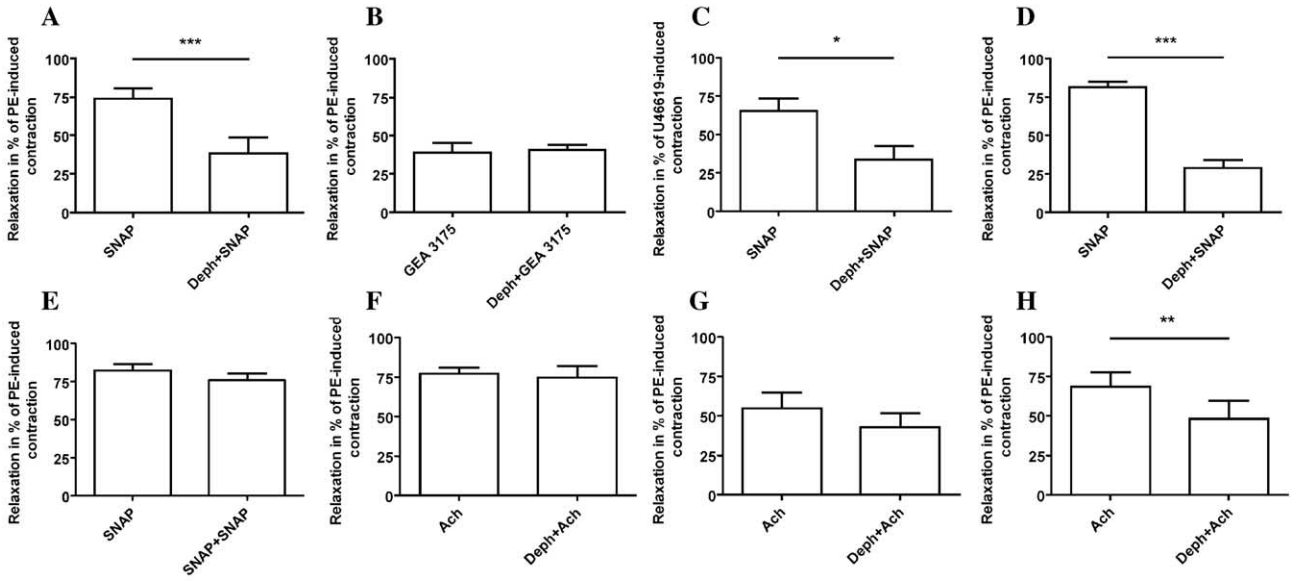

Fig. 4. Summarised effects of dephostatin on SNAP-, GEA 3175-, and acetylcholine-induced relaxation. Phenylephrine (PE; 1 μM)-precontracted arteries were exposed to dephostatin (Deph; 10 μM), SNAP (1 μM), GEA 3175 (0.1 μM), acetylcholine (Ach; 0.1 μM) alone, or combinations of the drugs. The effect of dephostatin (15 min exposure) on SNAP- and GEA-induced relaxation is shown in (A) and (B), respectively. The influence of dephostatin on SNAP-induced relaxation of arterial strips precontracted with the thromboxane A_2 analogue U46619 (0.01 μM) is summarised in (C). The role of the endothelium is shown in (D). In these experiments, the endothelium was removed and, thereafter, the arteries were exposed to dephostatin followed by SNAP. (E) shows the effect of repeated exposure of SNAP on phenylephrine-precontracted arteries. In these experiments, dephostatin was replaced by a low dose of SNAP (0.01 μM) followed by higher dose of SNAP (1 μM). (F)–(H) summarise the effect of dephostatin on acetylcholine-induced relaxation. In these experiments, dephostatin was introduced 15 (F), 30 (G), or 60 (H) prior to acetylcholine. SNAP-, GEA 3175-, and acetylcholine-induced relaxation was calculated as % of phenylephrine-induced contraction. The results are presented as mean±S.E.M., n=4–7. Statistical significance was tested with Student's t-test and is denoted *P<0.05, **P<0.01 and ***P<0.001.

dose, of SNAP (Fig. 4E). Taken together, the results presented in this section show that dephostatin induced vascular smooth muscle relaxation, but also antagonised the actions of the commonly used *S*-nitrosothiol SNAP. This effect was not observed by using the oxatriazole derivative GEA 3175. Furthermore, by increasing the length of incubation, dephostatin also reduced the effect of acetylcholine.

3.2. Analysis of cGMP accumulation in porcine iliac arteries

The cGMP content in blood vessel preparations was measured with radioimmunoassay. The arterial rings were pretreated with the phosphodiesterase inhibitor IBMX (100 μM) and then exposed to dephostatin (10 μM), SNAP (1 μM), and GEA 3175 (0.1 μM) alone, or dephostatin combined with SNAP or GEA 3175. We found that all compounds induced a significant increase in cGMP accumulation in porcine iliac arteries (Fig. 5). The magnitude of the response of dephostatin was, however, relatively small. The results also revealed that dephostatin significantly inhibited SNAP-induced cGMP accumulation. On the contrary, dephostatin did not affect GEA 3175-stimulated rises in the cGMP content. Thus, the results show that dephostatin directly and indirectly influenced on the synthesis of cGMP in the blood vessels.

3.3. Effects of dephostatin on protein phosphorylation

Previous studies have characterised dephostatin as an inhibitor of protein tyrosine phosphatases (Imoto et al., 1993). For that reason, we evaluated the effect of dephostatin on tyrosine-specific protein phosphorylation in porcine iliac arteries. Western blot analysis revealed that two proteins, around 85 and 65 kDa, were tyrosine phosphorylated in untreated blood vessel preparations (Fig. 6). Incubation with dephostatin (10 μM) did not significantly reinforce the degree of protein tyrosine

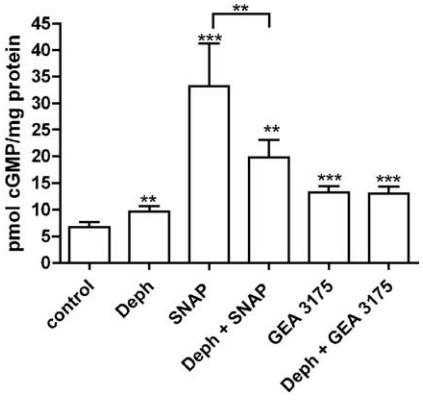

Fig. 5. Increases in cGMP levels in arterial preparations. Arterial rings were preincubated for 10 min with IBMX (100 μM) and then exposed to dephostatin (Deph; 10 μM) for additional 15 min. Thereafter, the vessel preparations were exposed to SNAP (1 μM) or GEA 3175 (0.1 μM) for 2 min. The levels of cGMP were determined with radioimmunoassay. Arteries exposed to dephostatin, SNAP, GEA 3175, or the solvent dimethyl sulfoxide (control) alone were analysed in parallel. Results are presented as mean values±S.E.M., n=6–14. Statistical significance was tested with ANOVA, followed by Newman–Keuls multiple comparison as post hoc test. Statistical significance are denoted **P<0.01 and ***P<0.001.

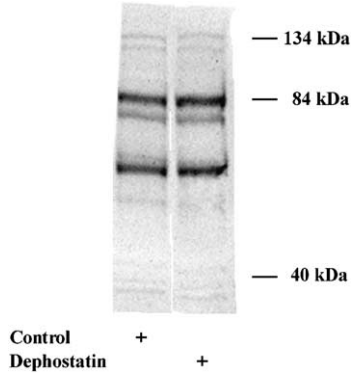

Fig. 6. Western blot analysis on tyrosine-specific phosphorylation of blood vessel proteins. Arterial rings were exposed to dephostatin (10 μM) or the equivalent volume of the solvent dimethyl sulfoxide (control) for 15 min. The degree of tyrosine phosphorylation of arterial proteins were analysed by Western blotting using an antibody directed towards phosphotyrosine residues. The figure shows one of 4 separate experiments.

phosphorylation. More specifically, densitometric analysis of the 65 kDA protein band revealed an 186±20% increase compared to background in untreated preparations (mean value ±S.E.M., $n=4$). Incubation with 10 μM of dephostatin for 15 min resulted in a 198±37% increase compared to background (mean value±S.E.M., $n=4$). Consequently, the results do not indicate for a significant effect of dephostatin on tyrosine phosphorylation of blood vessel proteins.

The 46/50 kDa protein VASP is a main substrate for cGMP-activated protein kinase in platelets and vascular smooth muscle cells. Fig. 7 shows that incubation of iliac arteries with SNAP (1 μM) or GEA 3175 (0.1 μM) resulted in an increase in serine^{239}-phosphorylated VASP. On the contrary, dephostatin (10 μM) did not produce a significant phosphorylation of VASP. In other series of experiments, arterial preparations were exposed to dephostatin (10 μM) for 15 min followed by SNAP or GEA 3175. As shown in Fig. 7, dephostatin diminished SNAP-induced phosphorylation of VASP. On the other hand,

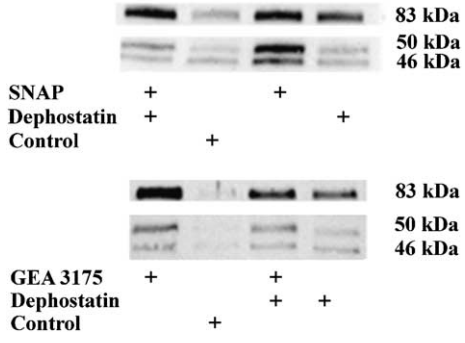

Fig. 7. Western blot analysis on serine^{239}-specific phosphorylation of vasodilator-stimulated phosphoprotein (VASP). Arterial rings were preincubated with dephostatin (10 μM) for 15 min and then exposed to SNAP (1 μM) or GEA 3175 (0.1 μm) for additionally 2 min. Blood vessel preparations exposed to the solvent dimethyl sulfoxide (control), dephostain, SNAP, or GEA 3175 alone were analysed in parallel. The samples were examined by Western blotting using an antibody directed towards serine^{239}-phosphorylated VASP. The figure illustrates the results from one of 3–9 separate experiments.

Table 1
Summarised effects of dephostatin, SNAP, and GEA 3175 on serine^{239}-phosphorylated VASP

Dephostatin	50 kDa		83 kDa		
	−	+	−	+	
Control	109±5	112±4	111±5	148±27	$n=9$
SNAP	149±13	121±4	413±1	327±8	$n=6$
GEA 3175	126±3	124±7	158±4	150±9	$n=3$

Iliac arteries were exposed to dephostatin (10 μM), SNAP (1 μM), GEA 3175 (0.1 μM) alone or in combinations. The samples were analysed by Western blotting using an antibody directed towards serine^{239}-phosphorylated VASP. The 50 and 83 kDa protein bands were quantified densitometrically using the LAS-1000 imaging analyser system. The values represent the mean values±S.E.M.

dephostatin did not affect GEA 3175-induced VASP phosphorylation. By using antibodies directed against serine^{239} phosphorylated VASP, the Western blots revealed a protein band around 83 kDa (Fig. 7). We found that SNAP, GEA 3175 and dephostatin increased the phosphorylation of the 83 kDa protein band. Fig. 7 indicates that dephostatin reduced the effect of SNAP, while the effect of GEA 3175 was unaffected. The influence of the compounds on phosphorylation of VASP is summarised in Table 1.

3.4. NO-scavenging action of dephostatin

The ISO-Mark II NO-sensor was used in order to detect NO in aqueous solutions. As shown in Fig. 8, SNAP (10 μM) rapidly decomposes to yield free NO molecules. Dephostatin (10 or 30 μM) alone did not induce a detectable NO response, but markedly affected the SNAP-induced release of NO. More specifically, pretreatment for 3 min with dephostatin accelerated the liberation of NO from SNAP, and increased the consumption of released NO molecules (Fig. 8A). The addition of dephostatin to a solution that already contained SNAP resulted in a rapid decline in the NO-dependent sensor signal. To further confirm this scavenging activity, solutions of authentic NO were exposed to dephostatin. In common with results obtained by using SNAP, dephostatin rapidly reduced the amount of free NO molecules (Fig. 8B). The capacity of dephostatin to liberate NO was also evaluated in sample containing a vessel preparation. However, even in the presence of an iliac artery, dephostatin (10–100 μM) failed to produce a NO response (inserted in Fig. 8).

4. Discussion

The nitrosoamine dephostatin, originally isolated from a strain of streptomyces, has been characterised as membrane permeable, competitive protein tyrosine phosphatase inhibitor (Imoto et al., 1993). Several published papers have shown that dephostatin modulates a great number of molecular and cellular responses. Generally, the underlying mechanism of drug action is considered to be due to increased phosphorylation of proteins on tyrosine residues. (Wijetunge et al., 1998; Voets et al., 1998; Murphy et al., 2003). Our results showed that treatment of porcine iliac arteries with dephostatin was not accompanied by

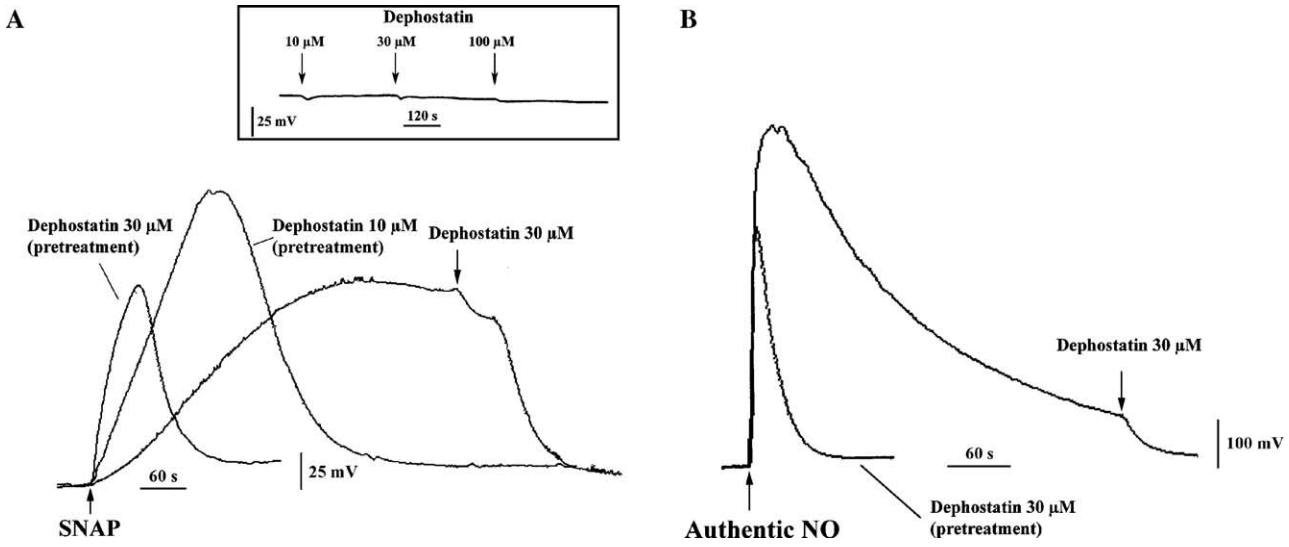

Fig. 8. Dephostatin increases the consumption of NO molecules. The NO response after adding 10 μM of SNAP (A) and authentic NO (B) was examined by using the Iso-Mark II NO-sensor. The experiments were analysed in the absence and presence of dephostatin (10–30 μM). Dephostatin was introduced prior to or after SNAP/NO (indicated by arrows). Inserted: the direct effect of dephostatin on NO release. The sample (containing an arterial strip) was stimulated with various concentrations (10–100 μM) of dephostatin (the additions are indicated by arrows). The original traces shown are representative for 3 different experiments.

increased tyrosine-specific protein phosphorylation. Despite that, dephostatin markedly affected vascular smooth muscle responses. Therefore, a main conclusion drawn, is that dephostatin is a protein tyrosine phosphatase inhibitor that exerts other mechanisms of drug action. In accordance, dephostatin has previously been found to increase the Ca^{2+} concentration in pancreatic acinar cells and that effect did not involve phosphatase inhibition (Lajas et al., 2000). From a general point of view, it is likely that the low doses (μM concentration range) and the short exposure time (minutes) that we used in the present investigation revealed protein tyrosine phosphatase inhibitor-independent actions of dephostatin. In fact, it has been shown that prolonged time of incubation (several hours) is a prerequisite for the detection of dephostatin-induced tyrosine phosphorylation (Suzuki et al., 2001; Wakino et al., 2001).

We suggest that dephostatin directly activates the NO/cGMP-signalling pathway in porcine iliac arteries. This statement is based on the following findings; i) dephostatin relaxed arterial preparations; ii) dephostatin elevated the cGMP levels in the vessel preparations; iii) the soluble guanylyl cyclase inhibitor ODQ abolished dephostatin-induced relaxation. Dephostatin contains a NO-moiety and, hence, may act as source for NO generation. For that reason, dephostatin may stimulate cGMP production and induce vasorelaxation. In comparison with other NO-containing compounds (i.e. SNAP and GEA 3175), the relaxation induced by dephostatin was very slow, the magnitude of relaxation was modest, and there was a marked variation in relaxatory response (approximately 30% of the blood vessel preparations did not respond with a detectable relaxation). One possible explanation could be that dephostatin must undergo intracellular metabolism to release bioactive NO. Theoretically, the requirement of biotransformation of the drug molecules may explain the slow onset of action of dephostatin.

It has been proposed that NO/cGMP-induced phosphorylation of serine^{239} on VASP correlates with relaxation of the blood vessel (Schafer et al., 2003). Indeed, our results showed that the *S*-nitrosothiol SNAP and the oxatriazole derivative GEA 3175 induced an increase in serine^{239}-phosphorylated VASP. However, Western blot analysis did not display a significant effect of dephostatin on VASP phosphorylation. On the other hand, Western blots indicated that dephostatin (in common with SNAP and GEA 3175) stimulated serine-specific phosphorylation of an 83 kDa protein. However, this protein is probably identical to a VASP-binding, zyxin-related protein found in porcine platelets (Reinhard et al., 1995). Thus, the interpretation of the results could be that exposure of arteries to dephostatin resulted in an increase in serine^{239}-phosphorylated VASP. Furthermore, phosphorylated VASP was almost exclusively located together with the zyxin-related protein. It is, however, to be noticed that the 83 kDa protein has been detected in some porcine cells, but has not been found in human tissues.

We also found that dephostatin differentially affected SNAP- and GEA 3175-stimulated arteries. More specifically, dephostatin antagonised SNAP-induced, but not GEA 3175-induced vasorelaxation. The inhibition of dephostatin on SNAP-induced relaxation was equally pronounced both when using another contractile agent (i.e. a thromboxane A_2 analogue), and when the endothelial layer was removed. In fact, dephostatin reduced all cellular and molecular aspects (i.e. elevation of cGMP and increased serine-specific phosphorylation of VASP) of SNAP-stimulated blood vessels. We found that dephostatin increased the consumption of SNAP-derived NO. This scavenging phenomenon was confirmed by using authentic NO. Based on these findings, we propose that the nitrosoamine dephostatin is an effective scavenger of NO molecules. Taken together, our findings suggest that dephostatin increases the degradation of NO, and on that account reduces SNAP-stimulated cGMP production, cGMP-dependent protein phosphorylation and the subsequent vasorelaxation. However, an intriguing finding

was that dephostatin also caused a more rapid and larger increase in NO release from SNAP. The liberation of NO from *S*-nitrosothiols may be accelerated by Cu^{2+}, cysteine, and enzymatic catalysis (Zhang and Hogg, 2005). Our experiments were performed in a cell-free buffer and, hence, mechanisms involving cellular enzymes and metal ions are presumably not involved. It has been reported that dephostatin stimulates oxidation processes and on that account affects cellular responses (Lajas et al., 2000). Whether such a drug mechanism explains the accelerated release of NO from SNAP remains to be ascertained. None the less, the present results show that the nitrosoamine dephostatin has a dual effect on the NO/cGMP signalling pathway in porcine iliac arteries. Amperometric measurements of NO revealed that dephostatin alone did not liberate detectable amount of NO. The most reliable explanation is that the drug antagonises its own action in the tissue; more specifically, intracellular biotransformation would be required for NO release (see the discussion above), while intact dephostatin molecules could act as NO scavengers.

We also observed that dephostatin reduced acetylcholine-mediated vasorelaxation. However, a longer incubation period was a prerequisite to detect the inhibitory effect of dephostatin. In an organ bath, NO is released by acetylcholine-stimulated endothelial cells both in the buffer and into the smooth muscle layer. Therefore, it is likely that dephostatin must accumulate in the myoendothelial space in order to inhibit endothelium-dependent relaxation. In accordance, it is well known that classical NO-scavengers may need hours of incubation to affect endothelium-mediated relaxation. The oxatriazole derivative GEA 3175 is characterised as a lipid soluble and stable NO-containing compound (Kankaanranta et al., 1996; Asplund Persson et al., 2004). Consequently, it is plausible that GEA 3175 accumulates in the biological membrane and/or other intracellular microenvironment. Thus, GEA 3175 may be more protected from the scavenging actions of dephostatin. Moreover, GEA 3175 does not release detectable amount of NO in an aqueous solution (Asplund Persson et al., 2004). Most likely, this property of GEA 3175 contributes to the insensitivity towards dephostatin. The *S*-nitrosothiol SNAP has been extensively used to study molecular, cellular, and physiological, aspects of NO (Feelisch, 1998; Al-Sa'doni et al., 2000; Napoli and Ignarro, 2003). Oxatriazole derivatives are less well characterised, but based on our findings it is likely that these agents are far more resistant to various NO-scavenging molecules. Stable, NO-containing substances, like GEA 3175, may for that reason induce a more long-lasting effect on cellular responses. Indeed, we have previously shown that GEA 3175 produced a remarkable long-acting relaxation of airway smooth muscle tissue (Johansson Rydberg et al., 1997).

In conclusion, the present paper shows that dephostatin directly activates the NO/cGMP-signalling pathway in porcine iliac arteries. Furthermore, we propose that dephostatin is a very effective scavenger of NO. These dual effects are apparently independent on inhibition of protein tyrosine phosphatases. Therefore, we conclude that, besides its action as a phosphatase-inhibitor, the nitrosoamine dephostatin may represent a novel and useful drug tool for elucidating the NO/cGMP-signalling pathway in different cells and tissues.

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