# Nicorandil attenuates cyclic strain-induced endothelin-1 expression *via* the induction of activating transcription factor 3 in human umbilical vein endothelial cells.

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## Abstract

Nicorandil is an adenosine triphosphate-sensitive potassium channel opener that combines an organic nitrate and a nicotinamide group which respectively confer to nicorandil the additional properties of being a nitric oxide (NO) donor and antioxidant; it also induces vasodilation, decreases the blood pressure, and protects the heart. However, the intracellular mechanism of nicorandil remains to be delineated. The aims of this study were to test the hypothesis that nicorandil alters strain-induced endothelin-1 secretion and NO production, and to identify the putative underlying signaling pathways in human umbilical vein endothelial cells (HUVECs). Cultured HUVECs were exposed to cyclic strain in the presence of nicorandil; endothelin-1 expression was examined by reverse-transcriptase-polymerase chain reaction and enzyme-linked immunosorbent assay. Activation of extracellular signal-regulated protein kinase (ERK), endothelial NO synthase (eNOS), and activating transcription factor (ATF)-3 were assessed by Western blot analysis. We show that nicorandil inhibited strain-induced endothelin-1 expression. Nicorandil also inhibited strain-increased reactive oxygen species formation and ERK phosphorylation. On the contrary, NO production, eNOS phosphorylation, and ATF3 expression were enhanced by nicorandil; however, L-NAME (an inhibitor of eNOS) and LY294002 (an inhibitor of phosphatidylinositol 3-kinase) inhibited nicorandil-increased ATF3 expression. Moreover, treatment of HUVECs with either an NO donor (NOC18; 3,3-bis[aminoethyl]-1-hydroxy-2-oxo-1-triazene) or an ATF3 activator (MG-132; carbobenzoxy-L-leucyl-L-leucyl-L-leucinal) resulted in repression of strain-induced endothelin-1 expression. Furthermore, L-NAME, and small interfering RNA transfection of eNOS also partially attenuated the inhibitory effect of nicorandil on strain-induced

endothelin-1 expression. We demonstrate for the first time that nicorandil inhibits strain-induced endothelin-1 secretion *via* an increase in NO and upregulation of ATF3 in HUVECs. This study provides important new insights into the molecular pathways that may contribute to the beneficial effects of nicorandil in the cardiovascular system.

*Keywords*: Nicorandil; Cyclic strain; Endothelin-1; Nitric oxide, Activating transcription factor 3

## 1. Introduction

Nicorandil (N-(2-hydroxyethyl)-nicotinamide nitrate) is a $K_{ATP}$ channel opener combining an organic nitrate and a nicotinamide group which respectively confer to nicorandil the additional properties of being a nitric oxide (NO) donor and an antioxidant; it is clinically used to treat ischemic heart diseases (Mason and Cockcroft, 2006). Nicorandil is known to act as an antioxidant, reacting with free radicals to prevent oxidative cell damage in several types of preparations (Mano et al., 2000). Nicorandil application was also reported to stimulate the production of NO by cultured vascular endothelial cells (Tajima et al., 2008). In addition, Horinaka *et al*. demonstrated that nicorandil increases cardiac endothelial NO synthase (eNOS) expression by activating $K_{ATP}$ channels in animal studies (Horinaka et al., 2001; Horinaka et al., 2004; Horinaka et al., 2006). However, the mechanism through which nicorandil exerts its protective effects is not fully understood. Endothelial dysfunction plays an important role in ischemia-reperfusion injuries (Misra et al., 2009). Key regulators of endothelial function are NO, a vasodilator (Cerra and Pellegrino, 2007) and endothelin-1, a vasoconstrictor (Thorin and Webb, 2010). Under physiological conditions, the highly controlled release of low amounts of NO by eNOS in the vascular endothelium causes blood vessels to dilate (Balligand et al., 2009). Endothelial NO exerts vasoprotective effects, and its deficiency is associated with increased cardiovascular risks in pathological situations such as diabetes, metabolic syndrome, hypertension and atherosclerosis (Cerra and Pellegrino, 2007). In addition, among the endogenous mediators of cardiovascular disorders, endothelin-1, a 21-amino-acid peptide, plays important roles in atherosclerosis, for which hypertension is an important risk factor, and in ischemic heart disease and stroke (Schiffrin, 2001).

Numerous studies showed that oxidative stress, represented by reactive oxygen species, is capable of significantly altering vascular function (Fearon and Faux, 2009). We demonstrated that intracellular reactive oxygen species mediate cyclic strain-induced endothelin-1 expression *via* the Ras/Raf/extracellular signal-regulated kinase (ERK) signaling pathway in endothelial cells (Cheng et al., 2001).

Activating transcription factor (ATF) 3, a member of the cyclic adenosine monophosphate response element binding (CREB) family of basic leucine zipper transcription factors, is a stress-inducible transcriptional repressor (Hashimoto et al., 2002). Gilchrist *et al.* (2006) identified ATF3 as a negative regulator of the inflammatory response in macrophages (Gilchrist et al., 2006). Recent studies showed that inhibition of endothelial matrix metalloproteinase (MMP)-2 expression by NO is mediated at least in part by the induction of ATF3 in endothelial cells (Chen and Wang, 2004). However, no existing studies addressed the interference of endothelin-1, NO, and ATF3 expression by nicorandil in vascular endothelial cells. In the present study, we investigated the effects of nicorandil on NO production, ATF3 expression, and strain-induced endothelin-1 expression; and identified the signaling protein kinase cascades that may be responsible for the putative effects of nicorandil.

## 2. Materials and methods

### 2.1. Materials

Imubind endothelin-1 enzyme-linked immunosorbent assay (ELISA) kits were purchased from Amersham-Pharmacia (Amersham, UK). 2',7'-Dichlorofluorescin diacetate (DCFH-DA) was obtained from Serva (Heidelberg, Germany). Nicorandil was kindly provided by Chugai (Tokyo, Japan). NOC18 (3,3-bis[aminoethyl]-1-hydroxy-2-oxo-1-triazene) and carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG-132) were obtained from Calbiochem (San Diego, CA, USA). All other chemicals of reagent grade were obtained from Sigma-Aldrich (St. Louis, MO, USA).

### 2.2. Endothelial cell culture

Human umbilical vein endothelial cells (HUVECs) were obtained from PromoCell (Heidelberg, Germany) as cryopreserved cells. After thawing, cells were plated in cultured flasks and cultured to confluence in MCBD 131 medium (PromoCell) containing 28 mM hydroxyethylpiperazine ethanesulfonic acid, 2% fetal calf serum, 0.1 ng/ml human recombinant epidermal growth factor, 1 ng/ml human recombinant basic fibroblast growth factor, 50 µg/ml gentamycin, 50 ng/ml amphotericin B, and 1 µg/ml synthetic hydrocortisone and supplemented with a mixture (PromoCell) containing endothelial cell growth factor and heparin. Cells were grown at 37 °C in a humidified 5% $CO_2$ atmosphere for 3~4 days. Confluent cultures between passages 2 and 10 were used for all experiments.

### 2.3. *In vitro* cyclic strain of cultured endothelial cells

Endothelial cells cultured on a flexible membrane base were subjected to cyclic strain

produced by a computer-controlled application of sinusoidal negative pressure as described previously (Cheng et al., 2001).

**2.4 RNA isolation and quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR)**

RNA was isolated using Totally RNA and Turbo-Dnase kits from Ambion (Austin, TX, USA). For the quantitative real-time RT-PCR analysis, 1 μg of total RNA was reverse-transcribed into complementary (c)DNA using the High-Capacity cDNA Archive Kit from Applied Biosystems (Foster City, CA, USA). Quantitative real-time RT-PCRs were performed using a QuantiTect SYBR Green PCR Kit from QIAGEN (Valencia, CA, USA) and analyzed on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Data were analyzed and the cycle threshold (ΔΔC) was calculated using SDS 2.2 software from Applied Biosystems. Primers (all obtained from Protech Technology Enterprise, Taipei, Taiwan) used for the human endothelin-1 messenger (m)RNA analysis were 5'-AAA CAG CAG TCT TAG GCG CTG A-3' (forward) and 5'-GAC ACA CTC TTT ATC CAT CAG GGA C-3' (reverse) (Reiter et al., 2010); for human β-actin mRNA analysis, they were 5'-CTG GCA CCC AGC ACA ATG AAG-3' (forward) and 5'-TAG AAG CAT TTG CGG TGG ACG-3' (reverse). Endothelin-1 mRNA expression was normalized to β-actin mRNA expression.

**2.5. Assay of endothelin-1 peptide secretion**

Endothelin-1 levels were measured in culture medium using a commercial enzyme-linked immunosorbent assay kit (ELISA; Amersham-Pharmacia, Amersham, UK). Results were normalized to the cellular protein content in all experiments and are expressed as a percentage relative to cells incubated with the vehicle.

### 2.6. Detection of intracellular reactive oxygen species

Measurement of intracellular reactive oxygen species formation in HUVECs was recorded by monitoring changes in dichlorofluorescein (DCF) fluorescence as described previously (Cheng et al., 2009).

### 2.7. Western blot analysis

A Western blot analysis was performed as described previously (Cheng et al., 2009). Membranes were blocked with 5% nonfat dry milk (NFDM) in PBS-0.1% Tween for 90 min and washed three times with PBS-0.1% Tween-1% NFDM; incubated with a 1:500 dilution of anti-phospho-ERK antibodies (New England BioLabs; Beverly, MA, USA), anti-ERK antibodies, anti-ATF3 antibodies (C-19) (Santa Cruz Biotechnology; Santa Cruz, CA, USA), anti-Ser1177 phospho-eNOS antibodies (Cell Signaling Technology, Beverly, MA, USA), or anti-eNOS antibodies (BD Bioscience; San Jose, CA, USA) in PBS-1% NFDM for 1 h. They were then washed three times with PBS-0.1% Tween-1% NFDM; and incubated with a 1:4,000 dilution of a horseradish peroxidase (HRP)-coupled appropriate secondary antibody (Pierce, Rockford, IL, USA) in PBS-0.1% Tween-1% NFDM and washed three times with PBS-0.3% Tween-1% NFDM.

### 2.8. Measurement of nitrate/nitrite levels

The culture medium was stored at - 70 ºC until used. After the medium was thawed, the sample was deproteinized with two volumes of 4 $^{o}$C 99% ethanol and centrifuged (at 3000 *g* for 10 min). These medium samples (100 μl) were injected into a collection chamber containing 5 % $VCl_3$. This strong reducing environment converts both nitrate and nitrite to NO. A constant stream of helium gas carried the NO to an NO analyzer (Seivers 270B NOA; Seivers Instruments, Boulder, CO, USA), where the NO was

reacted with ozone, resulting in the emission of light. The light emitted was proportional to the NO formed, and standard amounts of nitrate were used for calibration.

### 2.9. Short interfering (si) RNA transfection

eNOS siRNA (purchased from Santa Cruz Biotechnology) and mock control oligonucleotides were transfected using the Lipofectamine reagent according to the manufacturer's instructions. The final concentration of siRNAs for transfection was 100 nM. Transfected cells were washed and then incubated in new culture media for additional treatment as indicated and Western blot assays.

### 2.10. Statistical analysis

Data are presented as the mean ± standard error of the mean (S.E.M.). Statistical analysis was performed using Student's *t*-test or analysis of variance (ANOVA) followed by Dunnett's multiple-comparison test using Prism vers. 3.00 for Windows (GraphPad Software, San Diego, CA, USA). *P*-Values of $< 0.05$ were considered to be statistically significant.

## 3. Results

### 3.1. Effect of nicorandil on strain-induced endothelin-1 expression in endothelial cells

HUVECs cultured on flexible membrane bases were subjected to deformation to produce an average strain of 20%. We examined the effect of nicorandil on strain-increased endothelin-1 mRNA levels using a quantitative RT-PCR analysis. HUVECs under cyclic strain for 6 h showed a significant increase in the endothelin-1 mRNA level (data not shown); however, treatment with nicorandil (3~10 μM) significantly reduced strain-induced endothelin-1 expression (Fig. 1A). We then examined the effect of nicorandil on strain-increased endothelin-1 secretion. Endothelin-1 released into the culture media was measured. As shown in Fig. 1B, HUVECs under cyclic strain for 24 h showed increased endothelin-1 secretion, and nicorandil (3~10 μM) significantly inhibited the strain-increased endothelin-1 secretion. These data indicate that nicorandil inhibits strain-increased endothelin-1 secretion by endothelial cells.

### 3.2. Nicorandil inhibits strain-increased reactive oxygen species formation and ERK phosphorylation

Our previous study showed that cyclic strain increases reactive oxygen species formation in endothelial cells, which is involved in endothelin-1 induction (Cheng et al., 2001). Nicorandil is known to act as an antioxidant, reacting with free radicals to prevent oxidative cell damage in several types of preparations (Mano et al., 2000). We next examined whether nicorandil prevents strain-increased reactive oxygen species formation. The addition of nicorandil (3~10 μM) to cultured HUVECs significantly inhibited strain-induced reactive oxygen species formation as measured after strain

treatment for 1 h (Fig. 2A). To gain insights into the mechanism of action of nicorandil, we examined whether nicorandil affects intracellular protein kinase signaling pathways. Given that the ERK signaling pathway is involved in strain-induced endothelin-1 expression (Cheng et al., 2001), we further investigated whether nicorandil inhibits the ERK pathway in strain-treated endothelial cells. Treatment of cultured HUVECs with nicorandil (10 μM) or the antioxidant, N-acetylcysteine (NAC; 5 mM), also significantly inhibited strain-induced ERK phosphorylation (Fig. 2B). These findings demonstrate that nicorandil inhibits strain-increased intracellular reactive oxygen species levels and ERK phosphorylation in endothelial cells. These findings imply that nicorandil might inhibit a strain-activated ERK signaling pathway *via* attenuation of reactive oxygen species formation in endothelial cells.

### 3.3. Effects of nicorandil on NO production and phosphorylation of eNOS in HUVECs under cyclic strain

As nicorandil was reported to stimulate the production of NO by cultured vascular endothelial cells (Tajima et al., 2008), we detected nicorandil's effect on NO production and eNOS phosphorylation. Exposure of HUVECs to nicorandil time- and dose-dependently increased NO generation (Fig. 3A, B). Exposure of HUVECs to nicorandil (10 μM) for more than 15 min also increased eNOS phosphorylation (Fig. 3C). These findings reveal that nicorandil increased NO production and eNOS phosphorylation in endothelial cells.

### 3.4. Nicorandil induces ATF3 protein expression *via* NO production

To examine the effect of nicorandil on ATF3 expression in HUVECs, cells were incubated with nicorandil (10 μM) for 0.5~6 h, and the ATF3 protein expression was

analyzed by Western blotting. As shown in Fig. 4A, the induction of ATF3 was observed within 1 h and slightly decreased within 6 h after nicorandil treatment. ATF3 expression was induced after treatment with the ATF3 activator, MG-132 (5 μM) for 1 h. A previous study showed that NO-induced MMP-2 down-regulation was mediated by the induction of ATF3 (Chen and Wang, 2004). We examined the role of NO in mediating nicorandil-induced ATF3 expression in HUVECs. When HUVECs were pretreated with a specific inhibitor of eNOS (L-NAME, 100 μM) or phosphatidylinositol 3-kinase (PI3-K; LY294002, 5 μM), nicorandil-induced ATF3 expression was significantly suppressed (Fig. 4B). These results indicate that nicorandil induces ATF3 expression, and the PI3K-eNOS pathway may be involved in nicorandil-induced ATF3 expression by endothelial cells.

**3.5. Nicorandil results in the suppression of strain-induced** endothelin-1 **expression *via* NO production and ATF3 expression.**

To elucidate the inhibitory mechanism of nicorandil on strain-induced endothelin-1 expression, an NO donor (NOC18), ATF3 activator (MG-132), eNOS inhibitor (L-NAME), and siRNA for eNOS were applied to HUVECs. NOC18 (500 μM) and MG-132 (5 μM) attenuated the strain-induced endothelin-1 mRNA levels and endothelin-1 secretion of cells (Fig. 5A, B). The inhibitory effect of nicorandil on strain-induced endothelin-1 expression was partially reversed by L-NAME (100 μM) treatment and eNOS siRNA (100 nM) transfection (Fig. 5A, B). These results reveal the involvement of NO and ATF3 in nicorandil's effect on cyclic strain-induced endothelin-1 expression.

## 4. Discussion

The major new findings of this work are that nicorandil induces activation of eNOS, and the increase in NO appears to be involved in ATF3 expression and consequently results in suppression of strain-induced endothelin-1 secretion by endothelial cells. Nicorandil also inhibits strain-induced endothelin-1 expression in part *via* attenuation of reactive oxygen species formation in endothelial cells. Our previous studies indicated that hemodynamic forces, such as pressure-induced strain, can stimulate endothelin-1 gene expression (Cheng et al., 2009; Cheng et al., 2001). In addition, others and we have shown that cyclic-strain treatment of endothelial cells can induce intracellular reactive oxygen species generation (Cheng et al., 2009; Wung et al., 1997). Elevated reactive oxygen species levels are involved in the release of endothelin-1, and this gene induction can be attenuated by antioxidant pretreatment of cells (Cheng et al., 2001). Nicorandil is also a potent antioxidant (Mano et al., 2000). The results of our present study further demonstrate that nicorandil reduced strain-induced reactive oxygen species generation in endothelial cells. In particular, it was demonstrated that activation of ERK is redox-sensitive and that suppression of reactive oxygen species inhibits strain-induced endothelin-1 gene expression (Cheng et al., 2001). One possible explanation for the inhibitory effect of nicorandil on strain-induced endothelin-1 expression may thus be its ability to attenuate reactive oxygen species formation. Alternatively, nicorandil may inhibit strain-induced endothelin-1 gene expression by virtue of its increasing NO production. NO derived from eNOS was shown to play important roles in modulating endothelial functions including pro-apoptotic and anti-apoptotic effects (Cerra and Pellegrino, 2007). Those effects of NO on cells depend

on the quantity of NO and the expression of NO synthase isoforms in relevant tissues (Dimmeler and Zeiher, 1999). In the present study, we showed that NO contributes to ATF3 expression during nicorandil treatment of endothelial cells. Several studies demonstrated that ATF3 functions as a pro-apoptotic or anti-apoptotic regulator in cells (Kawauchi et al., 2002; Mashima et al., 2001). Although there are several studies on the effects of various stimuli on ATF3 expression and cellular functions, the effect of nicorandil on ATF3 expression and the mechanism underlying this regulatory effect of nicorandil in endothelial cells warrant further investigations. A specific inhibitor of PI3K (LY294002), which was shown to be the upstream signal for eNOS (Dimmeler et al., 1999), and L-NAME had inhibitory effects on nicorandil-induced ATF3 expression. Those results suggest that the PI3K-eNOS pathway is involved in nicorandil-induced ATF3 expression in HUVECs. Our findings also indicate that nicorandil suppresses strain-stimulated endothelin-1 expression *via* NO production. NO acting through soluble guanylyl cyclase and cGMP formation is a negative regulator of endothelin-1 gene induction (Gewaltig and Kojda, 2002). Our findings further support NO being a negative regulator of strain-induced endothelin-1 expression under nicorandil treatment.

## 5. Conclusions

In conclusion, the data obtained in the present study suggest that nicorandil-induced NO production and suppression of cyclic strain-induced endothelin-1 expression can be considered one of the mechanisms responsible for the protective effects of nicorandil in vascular vessels. These findings highlight the therapeutic potential of using nicorandil to treat vascular disorders. In light of our findings, nicorandil can be considered a potentially active compound for use in conditions where reactive oxygen species are implicated,

although clinical studies are needed to establish the usefulness of it in treating and preventing human diseases.

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**Conflict of Interest:** none declared.

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## Figure legends

Fig. 1. Nicorandil inhibits strain-induced endothelin-1 expression in HUVECs. Results are shown as the mean ± S.E.M. (n=6). * *P*<0.05 vs. the control (Ctrl); # *P*<0.05 vs. application of strain alone.

(A) Inhibition of strain-induced endothelin-1 mRNA by nicorandil. Cells were preincubated with nicorandil (1, 3, and 10 μM) and then stimulated by cyclic strain for 6 h or not stimulated.

(B) Nicorandil inhibited strain-induced endothelin-1 secretion. Cells were preincubated with nicorandil (1, 3, and 10 μM) and then stimulated with cyclic strain for 24 h or not stimulated.

Fig. 2. Effects of nicorandil on strain-increased reactive oxygen species formation and ERK phosphorylation.

(A) Effect of nicorandil on strain-induced reactive oxygen species generation. The fluorescence intensities in untreated control cells are expressed as 100%. Cells were preincubated with nicorandil (1, 3, and 10 μM) for 30 min and then stimulated with cyclic strain for I h or not stimulated. Results are shown as the mean ± S.E.M. (n=6). * *P*<0.05 vs. the control (Ctrl); # *P*<0.05 vs. application of strain alone.

(B) Inhibitory effect of nicorandil on strain-increased ERK phosphorylation. Cells were preincubated with nicorandil (10 μM) or N-acetylcysteine (NAC; 5 mM) and then stimulated with cyclic strain for 30 min. Phosphorylation of ERK was detected by Western blotting using an anti-phospho-ERK antibody. Densitometric analyses were performed with a densitometer. Data are shown as multiples of increase relative to the control groups. Results are shown as the mean ± S.E.M. (n=4). * *P*<0.05 vs. the control

(Ctrl); # *P*<0.05 vs. application of strain alone.

Fig. 3. Effects of nicorandil on NO production and eNOS phosphorylation.

(A) Time course of nicorandil-induced NO production in HUVECs. Cells were in a control condition (Ctrl) or treated with nicorandil (10 μM) for 10, 30, 60, or 120 min. Results are shown as the mean ± S.E.M. (n=6). * *P*<0.05 vs. the control (Ctrl).

(B) Cells from either the control or treated with nicorandil (1, 3, 10, and 30 μM) for 30 min. Results are shown as the mean ± S.E.M. (n=6). * *P*<0.05 vs. the control (Ctrl).

(C) Effects of nicorandil on the phosphorylation of eNOS in HUVECs. Cells were in a control condition (Ctrl) or treated with nicorandil (10 μM) for 5, 10, 15, 30, or 60 min. A Western blot analysis was performed to detect phospho-eNOS (Ser1177) and total eNOS. Results are shown as the mean ± S.E.M. (n=4). * *P*<0.05 vs. the control (Ctrl).

Fig. 4. Nicorandil induced ATF3 protein expression in endothelial cells. (A) HUVECs were treated with nicorandil (10 μM) for various time intervals. Cells were treated with MG-132 (5 μM) for 1 h as the positive control. (B) NO is involved in nicorandil-induced ATF3 expression. HUVECs were pretreated with an inhibitor of eNOS (L-NAME, 100 μM) or PI3 Kinase (LY294002, 5 μM) for 1 h followed by nicorandil (10 μM) for 1 h. Total cell lysates were immunoblotted with an anti-ATF3 antibody. Tubulin was used to show that equal amounts of proteins were loaded in each lane. Data are presented as the mean ± S.E.M. (n=3). * *P*<0.05 vs. the control (Ctrl).

Fig. 5. Nicorandil-suppressed strain-induced endothelin-1 expression is NO-dependent.

(A) Effects of NOC18, MG132, L-NAME, and eNOS siRNA on nicorandil-decreased strain-induced endothelin-1 mRNA expression. Cells were pretreated with NOC18 (500 μM) or MG-132 (5 μM) for 1 h. To inhibit the activity of eNOS, cells were pretreated with

L-NAME (100 μM) for 30 min or were transfected with eNOS siRNA. HUVECs were subjected to cyclic strain in the absence and presence of nicorandil for 6 h, and total RNA was extracted.

(B) Effects of NOC18, MG132, L-NAME, and eNOS siRNA on nicorandil-decreased strain-induced endothelin-1 secretion by HUVECs. Cells were pretreated with NOC18 (500 μM) or MG-132 (5 μM) for 1 h. To inhibit the activity of eNOS, cells were pretreated with L-NAME (100 μM) for 30 min or were transfected with eNOS siRNA. HUVECs were subjected to cyclic strain in the absence and presence of nicorandil for 24 h. Results are shown as the mean ± S.E.M. (n = 6). * $P$<0.05 vs. the control (Ctrl). $^{\#}$ $P$<0.05 vs. strain treatment. $^{+}$ $P$<0.05 vs. the nicorandil and strain treatment. Ctrl, untransfected control; M, mock control; seNOS, eNOS siRNA transfection.