Am J Physiol Heart Circ Physiol 287: H1977–H1986, 2004.
First published July 8, 2004; doi:10.1152/ajpheart.00323.2004.

Nitric oxide donor induces temporal and dose-dependent reduction of gene expression in human endothelial cells

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Submitted 1 April 2004; accepted in final form 24 June 2004

Braam, Branko, Remmert de Roos, Adele Dijk, Peter Boer, Jan Andries Post, Patrick P. C. W. Kemmeren, Frank C. P. Holstege, Hans A. R. Bluysen, and Hein A. Koomans. Nitric oxide donor induces temporal and dose-dependent reduction of gene expression in human endothelial cells. *Am J Physiol Heart Circ Physiol* 287: H1977–H1986, 2004. First published July 8, 2004; doi:10.1152/ajpheart.00323.2004.—The present study tested the hypothesis that acute increases in nitric oxide (NO) exert substantial influences on gene transcription in endothelial cells (ECs) via guanylyl cyclase (GC). Human umbilical veins ECs (HUVECs) were exposed to 0.1, 1, and 10 mM of sodium nitroprusside (SNP) for 4 h and to 1 mM SNP or 250 μM of (*Z*)-1[*N*-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]-diazen-1-ium-1,2-diolate (DETA-NONOate) for 2, 4, 8, and 24 h. Also, cells were exposed to DETA-NONOate in the presence and absence of the GC inhibitor 1*H*-[1,2,4]oxadiazolo-[4,3-*a*]quinoxalin-1-one (ODQ; 10 μM) for 4 h. RNA was isolated, reverse transcribed, Cy3 and Cy5 labeled, and analyzed using cDNA microarrays. Increasing doses of SNP predominantly depressed gene expression in HUVECs. Gene function was related to growth, adhesion, and cell structure. DETA-NONOate evoked a wave of expression changes (maximum at 4 h), with a remarkable downregulation of the transcription factors MSX1, RELB, and Egr-1. Both SNP- and DETA-NONOate-induced gene expression had faded after 24 h, despite continued elevation of cGMP in the medium. Coadministration of ODQ decreased many, but not all, of the transcriptional responses to DETA-NONOate. NO pronouncedly depressed EC gene expression, in particular of transcription factors. The observation that many, but not all, transcriptional changes induced by NO could be inhibited by inhibition of GC indicates the presence of GC-independent NO actions on gene expression. Thus EC gene expression responds to NO; however, the transcriptional response fades during prolonged exposure. This could allow the EC to respond to increased shear, without vigorous changes in gene expression.

microarray; nitrate tolerance; guanylate cyclase; Egr-1; V-Rel avian reticuloendotheliosis viral oncogene homolog B

SUBSTANTIAL EVIDENCE is now available on the actions of nitric oxide (NO) in the vasculature (4). The, by now already classical, paradigm is that NO, released from endothelial cells (ECs) in response to shear stress, acts on vascular smooth muscle cells (VSMCs) by activating soluble guanylyl cyclase (GC), resulting in cGMP formation and vasodilation (4). In addition, NO affects multiple other processes in VSMCs, i.e., inhibits proliferation (41) and modulates expression of genes with vasoactive function (19). NO also affects EC function in an autocrine fashion and thereby inhibits adhesion, inflammation, and generation of proliferative stimuli. Indeed, exogenously applied NO can affect transcription of adhesion molecules, such as ICAM-1 and E-selectin (23) and VEGF (11) in ECs that are activated with IL-1β or TNF-α. Interestingly, there is a large body of data available to support that NO actions in physiological settings are short lived, because of acute desensitization (5, 14) and slower adaptations of the GC pathway (14).

Despite these observations, information on the transcription response to NO in ECs and its dynamics is limited, whereas such transcriptional responses may have consequences for the integrity of the vascular wall on the long term. The activity of several transcription factors is modulated by alterations in NO levels, in particular early growth response-1 (Egr-1) (36), activating protein-1 (AP-1), Sp1, and nuclear factor (NF)-κB (7). Studies have mostly addressed transcription factor activity in other cells than ECs. Also largely unresolved is by which signaling pathways NO could affect gene expression in ECs. Potential mechanisms include the classical pathway via the generation of cGMP with subsequent activation of cGMP-dependent protein kinases (PKG) and modulation of the activity of phophodiesterases (PDEs) (33). However, NO will also alter the redox state of the cell and therefore could influence redox-sensitive transcription factors such as AP-1 and NF-κB independent of cGMP. Finally, NO directly nitrosylates proteins and thereby could also affect the action of transcription factors (20, 39). Investigation of the extent and pathway of transcriptional responses of ECs to NO could help to identify genes that prevent or cause EC activation and to understand further how the EC maintains its function during prolonged changes in ambient NO levels.

The hypothesis of the present study is that acute increases in NO exert substantial influences on gene transcription in ECs. It was addressed whether *1*) dose-response effects of NO on gene expression can be identified using microarray analysis in human umbilical vein ECs (HUVECs) in culture; *2*) such effects are short lived despite continued NO exposure; and *3*) transcriptional responses are mediated via cGMP. We chose to study gene regulation by microarray. This approach will leave open the translational and posttranslational modification processes and thus will only provide information about the sensing of NO of the cell.

MATERIALS AND METHODS

Isolation and culture of HUVECs. HUVECs were harvested from freshly obtained umbilical cords by use of the method described by

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Jaffe (20). Briefly, umbilical cords were rinsed with PBS and filled with 0.1% trypsine in PBS. After 30 min at 37°C, cells were harvested, spun down 10 min at 1,200 rpm, and resuspended into EGM-1 culture medium. The cells were then cultured at 37°C in humidified 95% air-5% CO_2 in conventional fibronectine-coated T-flasks. After reaching confluence, cells were transferred to T-flasks with RPMI (Invitrogen; Carlsbad, CA) medium containing 15% FCS (Invitrogen) and grown to confluence. Both culture media contained 0.5 U/ml penicillin and 0.5 mg/ml streptomycin. Cells that had gone through two passages were used for the experiments. One day before the experiments, the medium was replaced with RPMI now containing only 5% FCS.

Experimental design. The following experiments were performed. First, a dose-response curve was obtained for sodium nitroprusside (SNP; Sigma; St. Louis, MO). HUVECs were exposed to 0, 0.1, 1, and 10 mM SNP for 4 h. Second, a time-course experiment was performed: HUVECs were exposed for 2, 4, 8, and 24 h to 1 mM SNP. A similar experiment was also performed using 250 μM (*Z*)-1-[*N*-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA-NONOate; Cayman; Massy, France), which has a 20-h half-life at 37°C. In the SNP time-course experiment, 80% of the initial dose was added at 4 h in the 8-h experiment and at 4, 6, 12, 16, and 20 h in the 24-h experiment because of the half-life of SNP. Furthermore, HUVECs were exposed to 250 μM DETA-NONOate for 4 h in the presence and absence of 10 μM of the GC inhibitor 1*H*-[1,2,4]oxadiazolo-[4,3-*a*]quinoxalin-1-one (ODQ; Sigma). This concentration has been shown to effectively block the cGMP response to NO donors (16, 26). Control cells were exposed to vehicle (0.1% methanol). Finally, in four experiments, untreated cells from different flasks were compared. These control over control experiments are self-self experiments repeated four times to assess *1*) whether there are dye-specific effects on $\log_2$ ratios and *2*) what the variation is within the experiments. All experiments were repeated with cells from at least two separate primary cultures.

Harvesting of the cells and isolation of RNA. At the end of the experiment, cells were detached with 0.1% trypsine in PBS and collected in TRIzol reagent, which was stored at −20°C immediately. RNA was isolated according to the manufacturer's instructions, stored at −80°C until further processing, and quantitated using a spectrophotometer (Shimadzu Scientific Instruments; Columbia, MD).

Preparation of the probes and hybridization to the microarrays. Five micrograms of total RNA were added to 0.5 μg polyT_{1-18} primer (Invitrogen) in a total volume of 15 μl, heated to 70°C for 10 min, and immediately stored on ice. A mixture of 2 μl of 1 mM GTP, ATP, and CTP, 0.5 μl of TTP, 1.5 μl of aminoallyl-UTP (Sigma), 6 μl of first-strand buffer, 3 μl of 0.1 M DTT, 1 μl of reverse transcriptase (Invitrogen), and 1 μl of dH_2O was added. Reverse transcription was performed at 42°C for 120 min. After 2 min at 95°C, 10 μl of 1 M NaOH and 10 μl of 0.5 M EDTA were added to hydrolyze the RNA, and the sample was stored at 65°C for 30 min. pH was corrected using 25 μl of 1 M HEPES (pH 7.5). Sample cleanup was performed by applying the sample to 400 μl H_2O in a Microcon YM-30 filter (Millipore; Bedford, MA) and spun down at 10,000 rpm for 10 min. This procedure was repeated three times. The sample was then eluted by reversing the filter followed by spinning down at 2,000 rpm for 3 min. The volume was adjusted to 8 μl, after which 1 μl of 0.5 M sodium bicarbonate was added and 1.25 μl of Cy3 or Cy5 dye, dissolved in DMSO. The sample was stored at room temperature in the dark for 60 min. To quench the dye to the aminoallyl group in the cDNA, 4.5 μl hydroxylamine (4 M) was added, and the sample was stored in the dark for 15 min at room temperature. Unincorporated dye was removed by applying the samples to chromaspin 30 columns (DEPC version, BD Biosciences; Palo Alto, CA) using the manufacturer's instructions. The labeled cDNA was measured at this point; 50 ng in a volume of 10 μl was used for the hybridization.

Test samples (Cy3) were then mixed with the corresponding control sample (Cy5) for each specific experiment and added to 20 μl of 2× hybridization buffer [50% formamide, 10× SSC, 0.2% SDS, 200 μg/ml herring sperm (Invitrogen) DNA, and 200 μg/ml tRNA (Roche Diagnostics; Mannheim, Germany)]. Microarray slides were prehybridized in 100 ml prehybridization buffer containing 5× SSC, 25% formamide, 0.1% SDS, and 1% BSA (Sigma) for at least 45 min at 42°C. Samples were applied to the microarrays and covered with a coverslip, and hybridization was allowed to occur for 16 h at 42°C in the dark. For the present experiments, human cDNA arrays were obtained from the Microarray Centre of the University Health Network. These slides contained cDNA representing 1,700 human genes spotted in duplo. It should be emphasized that the set has been sequence verified by the Toronto Microarray Centre. Both the full list of represented genes and the sequences of the spotted cDNA can be found at the Toronto Microarray Centre website (http://www.microarrays.ca).

Microarray scanning and image analysis. Microarray slides were scanned using Scanarray 4000 system and software (Perkin-Elmer; Boston, MA) at 10 μm pixel size. Laser intensity was varied between 90% and 100% and photomultiplier tube sensitivity between 60% and 70% to optimize signal to background intensities. Stored images were analyzed using Imagene (Biodiscovery, Marina Del Rey) software.

RT-PCR to confirm array data. Reverse transcription was executed using an RT kit following the manufacturer's instructions (Lifetech 11904-018, Invitrogen). Primer pairs for MSX1 were forward: 5′-GCTAGAGGCCATGTCTCCTG-3′, reverse: 5′-CCCCAGAGCAAATGTTTTGT-3′; for RELB foward: 5′-TCCCAACCAGGATGTCTAGC-3′, reverse: 5′-AGCCATGTCCCTTTTCCTCT-3′; and for Egr-1 forward: 5′-CCGCAGAGTCTTTTCCTGAC-3′, reverse: 5′-TGGGTTGGTCATGCTCACTA-3′. PCR was performed with 30 cycles for MSX1 and 35 cycles for Egr-1 and RELB (30 s at 95°C, 30 s at 55°C, and 1 min at 72°C). PCR products were visualized on a 2% agarose gel with ethidium bromide.

cGMP levels in the medium and in cells. cGMP was measured using a kit, following the instructions of the manufacturer (Amersham Biosciences; Buckinghamshire, UK). cGMP was determined in the medium of cells exposed to 1 mM SNP for 2, 4, 8, and 24 h and compared with the medium of untreated cells. cGMP levels were also determined in medium and in the cells of cells exposed to 0, 4, or 24 h of 250 μM DETA-NONOate and in medium and cells exposed to 250 μM DETA-NONOate for 4 h in the presence of 100 μM of the GC inhibiter ODQ.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide viability assay. Cell viability was evaluated by determining metabolic activity using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma) (27). Cells were incubated for 4 or 24 h with 250 μM DETA or 24 h with 500 μM H_2O_2 in growth medium. At the end of the incubation, MTT was added to the culture medium from a 10× stock to a final concentration of 0.5 mg/ml. The cells were subsequently further incubated for another 90 min, and micrographs were taken using an inverted phase-contrast microscope (Zeiss Axiovert 220, equipped with a Hitachi HV-D30 camera) to visualize individual cell viability. Subsequently, cells were washed and dissolved in 0.04 N HCl in isopropanol, and the MTT signal was measured by determining the absorbance at 570 nm using a Benchmark Microplate reader (Bio-Rad).

Calculations, statistics, and Minimum Information About a Microarray Experiment compliance. Data of the Cy3 channel were normalized to the median intensity of the Cy5 channel. Data points were only used if the signal intensity exceeded the background plus two times the SD of the background and <50,000. Hierachical clustering was applied to both the dose-response and time-course data (see below) by use of the software developed by Jaak Vilo (Expression Profiler; http://ep.ebi.ac.uk/EP/). We used the average of the SD (0.28) of the four experiments where untreated samples were compared with untreated samples from RNA of the same batch of cells. One method to obtain a cut off is to use such a value to calculate a 95% confidence limit ($2.5 \times 0.28 = 0.7$). Besides, the average signal

of the control over control experiment ± 2SD of the four control over control experiments was evaluated and related to the observed changes as indicated in the text. Data and procedures were submitted in Minimum Information About a Microarray Experiment (MIAME) format (9) to the European Bioinformatics Institute. Experiments accession numbers are listed in the webappendix (www.nephrogenomics.net/data/appendices/ECexoNO-AJP2004).

RESULTS

cGMP levels and viability of the cells. One millimolar SNP (with the addition of extra SNP as detailed in MATERIALS AND METHODS) increased cGMP in the medium to about two times baseline; within 2 h, the level increased, and this was maintained throughout the 24-h period. Figure 1 shows the relative levels of cGMP in the medium and in cells exposed to 250 μM DETA-NONOate for 4 and 24 h. cGMP levels in the medium and in the cells were increased after 4 h of DETA-NONOate and remained elevated after 24 h. ODQ effectively inhibited the increase in cGMP in the cells and medium. There were no significant time-dependent changes in the amount of RNA extracted from the cells in the dose-response, time course, or ODQ experiments. The cells did not display any morphological abnormalities when inspected. In the microarray analysis, we did not find genes specifically related to apoptosis being induced after 24 h. Morphological evaluation clearly shows metabolic activity in both control cells and DETA-NONOate-treated cells. Subsequent quantification of the metabolic activity, as shown in Fig. 1, confirmed this observation.

Gene expression responses to increasing doses of SNP. First, we assessed gene expression profiles of HUVEC exposed to 0.1 (3 arrays from 3 independent experiments), 1.0 (3 arrays from 3 independent experiments), and 10 (3 arrays from 3 independent experiments) mM SNP for 4 h compared with

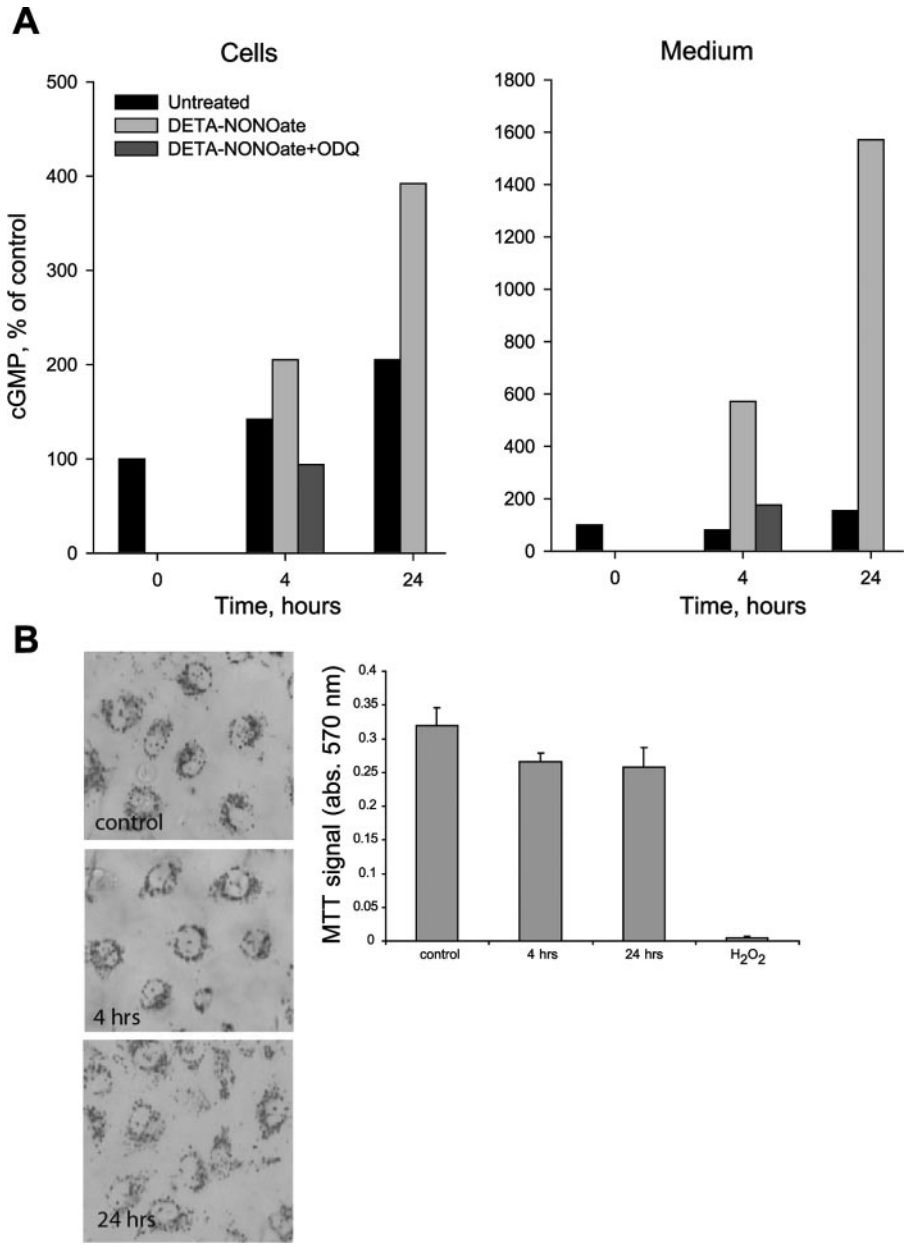

Fig. 1. *A*: intracellular cGMP levels and cGMP levels in the medium of human umbilical vein endothelial cells (HUVECs) exposed to 250 μM (*Z*)-1[*N*-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA-NONOate; DETA) for 4, 8, and 24 h compared with control, untreated cells. The addition of 100 μM of the guanylyl cyclase (GC)inhibitor1*H*-[1,2,4]oxadiazolo-[4,3-*a*]quinoxalin-1-one (ODQ) effectively blocked cGMP accumulation in cells and medium. *B*, *left*: micrographs showing individual cellular metabolic activity (resulting in purple precipitates) upon control or 250 μM DETA (4 and 24 h) incubation. *Right*, bar graph showing quantification of the metabolic activity after the various treatments. For comparison, the effect of 500 μM H_2O_2 is shown, which results in loss of metabolic activity and cell viability. MTT, 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide.

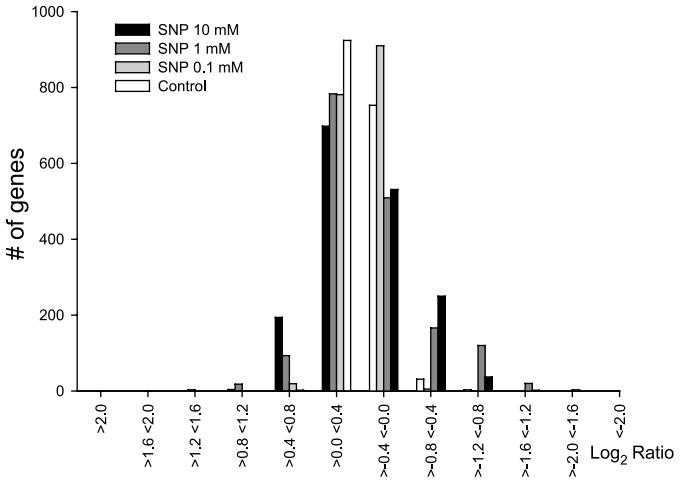

Fig. 2. Distribution of the $\log_2$ ratios of HUVECs exposed to increasing doses of sodium nitroprusside (SNP) for 4 h compared with ratios obtained from control (Con) untreated cells. The highest concentration of SNP resulted in the highest number of ratios deviating from 0; more genes were depressed than activated by SNP treatment.

untreated control cells from the same experiments. Figure 2 shows the distribution of the $\log_2$ ratios for each dose and for untreated cells compared with untreated cells from other flasks. The number of genes with a $\log_2$ ratio deviating from zero (no change) increased with increasing doses of SNP. Furthermore, the changes were relatively small and more genes decreased than increased. Figure 3 displays the result of hierarchical clustering. Genes are only displayed if in one of the four conditions, the $\log_2$ ratio was >0.7 or <-0.7. The red color depicts a positive $\log_2$ ratio, and the green color depicts a negative $\log_2$ ratio. The dendrogram depicts the similarity of the clusters. From this analysis, it was obvious that not all genes show a consistent increase or decrease with increasing doses of SNP. More genes were induced than repressed.

To further dissect the expression response, genes with a $\log_2$ ratio at the highest dose of SNP <-0.7 were grouped in biological function groups and are listed in Table 1. Table 1 also indicates the variation in the $\log_2$ ratios of the control over control experiments. From this analysis, it was clear that SNP decreased the expression of several genes related to growth, adhesion, cell structure, and transcription. The number of genes that increased was rather small, and no clear biological function was associated with these genes (not shown).

Time-course experiment with 1 mM SNP. Next, HUVECs were exposed to the intermediate dose, 1 mM, of SNP for 2, 4, 8, and 24 h in two independent experiments. Only few genes responded to 1 mM SNP at other time points than the above-mentioned 4 h. After 24 h, the effects of SNP had almost completely disappeared. Because of the relatively low number of genes responding to the intermediate dose of SNP, we proceeded to test the response to 250 μM DETA-NONOate (2 independent experiments), an NO donor with a long-half life. Figure 4 displays the distribution of the $\log_2$ ratios of genes that changed with 1 mM SNP versus 250 μM DETA-NONOate at 4 h. In contrast to 1 mM SNP, application of 250 μM DETA-NONOate showed a more prominent response.

Figure 5 displays the global changes as visualized by hierarchical clustering, as obtained during the time-course experiment with 250 μM DETA-NONOate. The red color depicts a positive $\log_2$ ratio, and the green color depicts a negative $\log_2$ ratio. As can be appreciated from Fig. 5, the effects of DETA-NONOate were maximal after 4 h and also virtually absent after 24 h. A list of all the observed ratios is available online (see www.nephrogenomics.net/data/appendices/ECexoNO-AJP2004); the supplemental file contains all data where one absolute $\log_2$ ratio exceeds 0.7. Comparison of the patterns of hierarchical clustering of the SNP (Fig. 3) and DETA-NONOate (Fig. 5) experiments indicates stronger effects of the latter on gene expression. This can also be appreciated by comparison of the $\log_2$ ratios in Tables 1 and 2. Table 2 shows genes that

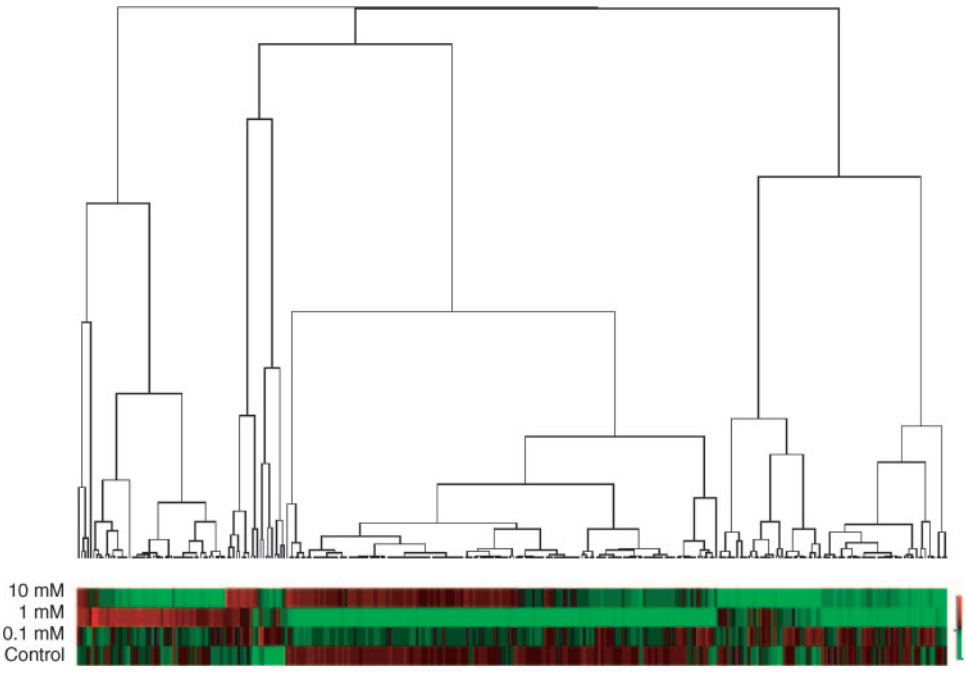

Fig. 3. Hierarchical cluster analysis of the SNP dose-response experiment. Only genes are displayed if in one of the four conditions the $\log_2$ ratio was >0.7 or <-0.7. Induction is represented in red, and repression is represented in green.

Table 1. *Gene expression responses to increasing doses of SNP*

Symbol	Clone ID	Name	SNP, mM			Control Over Control			
			0.1	1	10	Mean	SD	Mean − 2SD	Mean + 2SD
Proliferation									
PDGFB	343320	Platelet-derived growth factor-β polypeptide	0.10	−0.36	−1.36	−0.16	0.21	−0.57	0.25
TGFBR2	469876	Transforming growth factor, β-receptor II	−0.10	−0.24	−1.12	−0.01	0.43	−0.87	0.85
RASA1	243630	RAS p21 protein activator 1	−0.03	−0.11	−1.33	−0.71	0.49	−1.69	0.28
GAS1	341345	Growth arrest-specific 1	−0.21	−0.25	−0.82	−0.31	0.30	−0.90	0.28
CHN1	44590	Chimerin (chimaerin) 1	0.31	0.12	−0.72	−0.33	0.31	−0.96	0.30
EDNRA	487341	Endothelin receptor type A	−0.22	−0.74	−0.76	0.14	0.33	−0.51	0.80
ENG	310040	Endoglin (Osler-Rendu-Weber syndrome 1)	−0.08	−0.30	−0.62*	0.00	0.04	−0.08	0.08
PGF	470635	Placental growth factor, VEGF-related protein	0.04	0.12	−0.63**	−0.26	0.21	−0.67	0.16
Cell cycle									
CCNG1	48670	Cyclin G_1	0.13	−0.76	−1.17	−0.50	0.92	−2.34	1.33
CDK8	39255	Cyclin-dependent kinase 8	−0.46	0.36	−1.04	−0.27	0.31	−0.89	0.34
Structure and adhesion									
SELE	186132	Selectin E	0.12	−0.37	−1.08	−0.22	0.16	−0.54	0.09
KRT8	207999	Keratin 8	−0.07	0.19	−1.05	−0.21	0.29	−0.79	0.37
TUBB4	184151	Tubulin, β_4	−0.02	0.65	−0.91	0.06	0.14	−0.21	0.33
ITGA6	159512	Integrin, α6	0.27	−1.13	−0.90	0.19	0.12	−0.05	0.44
VCAM1	44477	Vascular cell adhesion molecule 1	0.09	0.37	−0.89	−0.11	0.28	−0.67	0.45
ANXA8	505239	Annexin A_8	−0.25	−0.33	−0.82	−0.19	0.21	−0.61	0.23
TUBB5	180858	Tubulin, β_5	0.00	0.39	−0.73	0.01	0.32	−0.63	0.64
Intercellular matrix									
MMP12	257581	Matrix metalloproteinase 12	−0.43	0.20	−1.14	−0.12	0.12	−0.36	0.12
MME	446372	Membrane metalloendopeptidase	0.71	−0.14	−0.87	0.12	0.24	−0.37	0.61
MMP11	300992	Matrix metalloproteinase 11	−0.56	−0.60	−0.67	0.09	0.23	−0.36	0.54
Transcription									
ZNF7	429072	Zinc finger protein 7	−0.18	0.17	−1.09	0.04	0.19	−0.33	0.41
ATF7	196174	Activating transcription factor 7	0.10	0.33	−0.86	−0.18	0.06	−0.29	−0.07
ZNF195	48413	Zinc finger protein 195	−0.01	0.61	−0.82	−0.29	0.23	−0.76	0.17
ZNF136	343096	Zinc finger protein 136	−0.08	0.60	−0.72	0.08	0.10	−0.12	0.28
VHL	150801	von Hippel-Lindau syndrome	0.09	−0.68	−0.71	−0.14	0.44	−1.03	0.74
VDR	344295	1,25-dihydroxyvitamin D_3 receptor	−0.02	0.22	−0.68	0.04	0.14	−0.23	0.31
Otherwise interesting									
F3	136590	Coagulation factor III (thromboplastin, tissue)	0.53	−1.98	−0.86	−0.39	1.02	−2.42	1.64
FOLR2	469329	Folate receptor 2 (fetal)	0.06	−0.24	−0.81	−0.20	0.31	−0.83	0.43
DHFR	294643	Dihydrofolate reductase	−0.06	−0.26	−0.64	0.16	0.12	−0.09	0.40

Shown are genes with absolute $\log_2$ ratios of 0.7 and higher at the highest dose of sodium nitroprusside (SNP). *Note the very tight $\log_2$ ratios in the control over control experiment; **DETA-NONOate induced a $\log_2$ ratio at 4 h of −0.74.

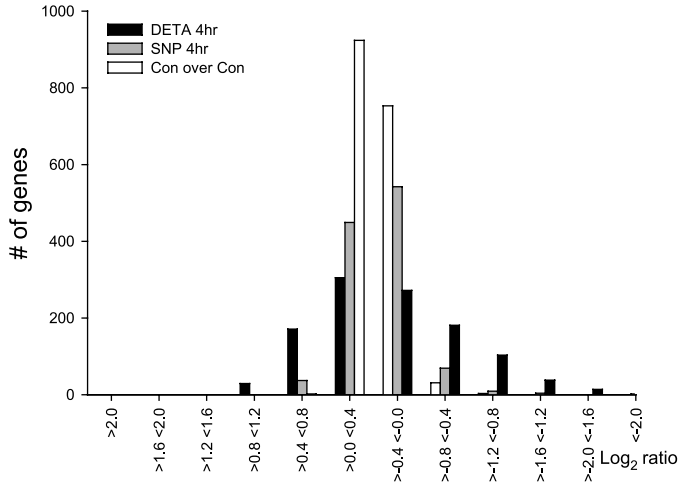

Fig. 4. Comparison of the distribution of the $\log_2$ ratios of HUVEC exposed to 1 mM SNP and 250 uM DETA-NONOate for 4 h compared with untreated cells. DETA-NONOate administration resulted in a higher number of genes displaying $\log_2$ ratios deviating from 0.

displayed a ratio exceeding 0.7 and belonged to the group of genes with a function involving transcription modulation. It became clear from this analysis that an important number of transcription factors were altered by exogenous NO. Figure 6 shows the RT-PCR of the transcription factors that showed the most prominent change at 4 h: Egr-1, MSX1, and v-Rel avian reticuloendotheliosis viral oncogene homolog B (RELB). The change in FOXF2 was tested but could not be confirmed with RT-PCR; however, the variance in the control over control experiment indicates wide variation in signal on the array. The average $\log_2$ ratios of the other genes at 4 h after application of either SNP or DETA-NONOate were consistent with the RT-PCR.

Consequences of blockade of GC on transcriptional responses to DETA-NONOate. In a separate experiment, we tested whether part of the actions of NO are mediated by a cGMP-independent pathway. Cells were exposed to 250 μM DETA-NONOate for 4 h in the presence or absence of ODQ ($n = 2$ for each). In this experiment, 250 μM DETA-NONOate administration for 4 h increased the expression of 40 and decreased the expression of 100 genes. During concomitant ODQ treatment, only seven genes exceeded a $\log_2$ ratio of 0.7 and seven genes had a $\log_2$ ratio of <-0.7. It should be

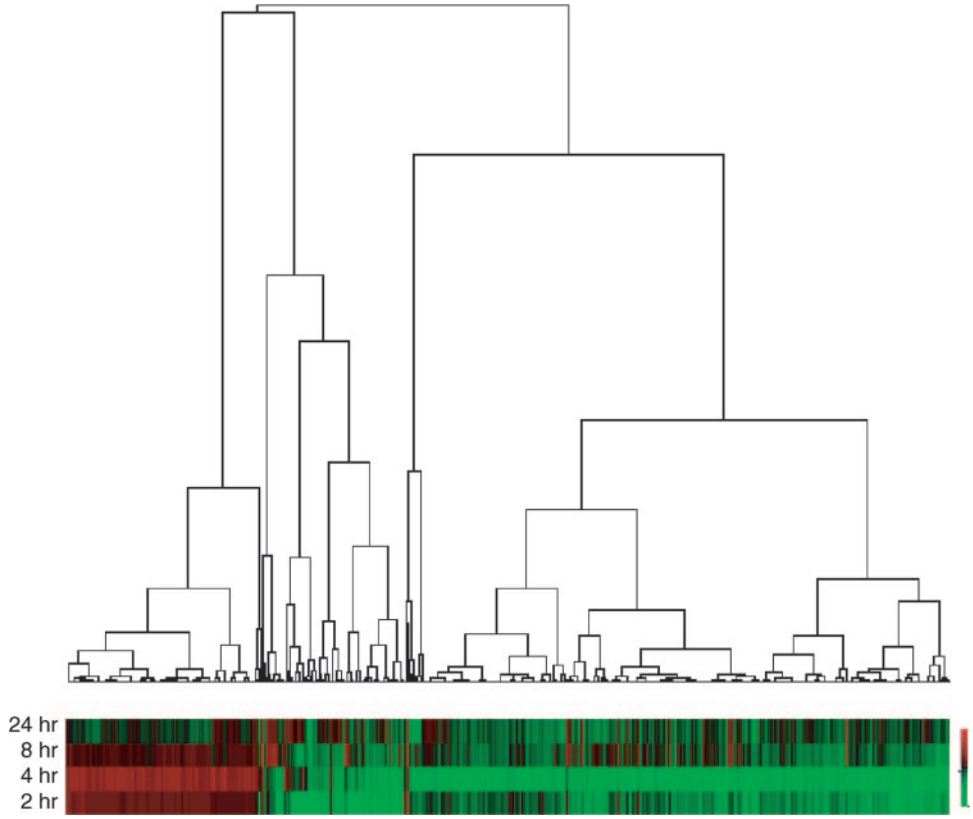

Fig. 5. Hierarchical cluster analysis of the time course experiment in which HUVECs were treated with 250 μM DETA-NONOate for 2, 4, 8, and 24 h. Expression changes were most profound at 4 h of treatment and faded at 8 and 24 h respectively. Only genes are displayed if in one of the four conditions the $\log_2$ ratio was >0.7 or <-0.7. Induction is represented in red, and repression is represented in green.

remarked that of the 100 genes depressed by DETA-NONOate, only 68 displayed a $\log_2$ ratio 0.7 higher during concomitant ODQ administration. Figure 7 summarizes the extent of changes in expression: DETA-NONOate again caused clear changes in expression; a part of the changes was inhibited by ODQ. Table 3 lists the changes induced by DETA-NONOate and the effect of addition of ODQ (only genes of which the $\log_2$ exceeded 1 or -1 for DETA-NONOate are displayed).

DISCUSSION

The present study was undertaken to investigate gene expression changes in human ECs on exposure to NO. Increasing dosages of the NO donor SNP resulted in increasing number of differentially expressed genes. As may be expected, more genes were repressed than activated. Among the decreased genes, several functional groups could be recognized that are closely related to the function of NO, in particular genes with antiproliferation and antiadhesion characteristics. Both SNP and the long-acting NO donor DETA-NONOate induced changes in gene expression that faded after 24 h, despite continued elevated cGMP in the medium. Changes observed with DETA-NONOate were more prominent than those with SNP. A number of transcription factors were inhibited by exposure to NO. Finally, the actions of DETA-NONOate on EC gene expression were largely, but not completely, inhibited by the inhibition of GC, indicating that most, but not all, actions of NO are cGMP mediated.

Exposing HUVECs to NO induced dose-dependent changes in gene expression. SNP administration resulted in decreases in gene expression in genes coding for growth factors, cell structure, and adhesion molecules. Some of these actions of NO on ECs have been documented by others in earlier experiments. Exogenous NO has been shown to inhibit shear stress-induced PDGF (2), endothelin-1 (31), E-selectin, and ICAM-1 (42) gene expression in ECs. Furthermore, exogenous NO has been demonstrated to inhibit LPS-induced tissue factor expression (RT-PCR) (32). New observations include a slight decrease in the expression of endoglin and placental growth factor. Endoglin is a component of the transforming growth factor (TGF)-β receptor complex and can interact with the TGF-β I and II receptor (13). The TGF-β II receptor has been implicated in induction of angiogenesis in hepatoma (21). Placental growth factor is a VEGF family member that is proangiogenic like VEGF (25). As yet, no clear studies on the interaction with NO have been reported for these two genes.

After 4 h, DETA-NONOate decreased the gene expression of many transcription factors by a $\log_2$ ratio of >0.7, while only increasing the expression of seven such factors. The strongest decrease was found for Egr-1, which is induced in the early response of ECs to shear stress (8). This response is inhibited by NO donors (10, 36). The present data confirm that Egr-1 is depressed by NO, even in the absence of stimulation by shear stress. Besides Egr-1, we confirmed depressed expression of two other genes: MSX1 [also referred to as homolog of *Drosophila* muscle segment homeo box 1 or homeo box 7 (HOX7)] and RELB with RT-PCR. MSX1 is a transcriptional repressor belonging to the homeobox factors and has been implicated in cell cycle regulation (18). The observed regulation of RELB, one of the NF-κB family members, in ECs is novel, because this protein has been primarily studied in lymphoid cells (3). Inspection of the genes repressed by SNP and DETA-NONOate discloses a number of known target

Table 2. *Genes related to transcription regulation that were changed upon DETA-NONOate application during the time course experiment*

Clone ID	Symbol	Time, h 2	4	8	24	Description
295093	SPIB	0.37	0.89	0.22	−0.05	Spi-B transcription factor (Spi-1/PU.1 related)
321607	FOXO1A	0.36	0.89	0.24	−0.28	Forkhead box O1A (rhabdomyosarcoma)
132373	BNC	0.25	0.80	0.11	−0.04	Basonuclin
417759	TAF10	0.32	0.78	0.03	−0.32	TAF10 RNA polymerase II, TATA box binding protein associated factor, 30 kDa
502990	ZNF208	0.26	0.75	0.23	0.04	Zinc finger protein 208
267600	STAT6	0.32	0.72	0.21	0.05	Signal transducer and activator of transcription 6, interleukin-4 induced
46180	RARB	−0.87	−0.63	−0.42	0.06	Retinoic acid receptor-β
41796	NRIP1	−0.93	−0.63	−0.60	−0.12	Nuclear receptor interacting protein 1
135050	HOXB5	−0.38	−0.70	−0.33	−0.11	Homeo box B_5
469768	ZFP36L2	0.16	−0.72	0.40	0.19	Zinc finger protein 36, C3H type-like 2
160060	PPARG	−0.41	−0.73	−0.16	−0.75	Peroxisome proliferative activated receptor-γ
290949	TRIP-Br2	−0.06	−0.80	−0.29	−0.68	Transcriptional regulator interacting with the PHS-bromodomain 2
38452	NR4A2	−0.48	−0.81	−0.47	−0.37	Nuclear receptor subfamily 4, group A, member 2
182473	NFKB2	−0.01	−0.82	−0.04	−0.08	Nuclear factor of κ-light polypeptide gene enhancer in B-cells 2 (p49/p100)
470591	USF2	−0.06	−0.88	−0.06	0.02	Upstream transcription factor 2, c-*fos* interacting
221092	GABPB1	−0.04	−0.92	0.27	−0.09	GA binding protein transcription factor, β-subunit 1 (53 kDa)
489133	GTF2A2	−0.49	−0.95	−0.07	−0.02	General transcription factor IIA, 2 (12 kDa subunit)
487797	DR1	−0.25	−0.97	0.34	0.24	Downregulator of transcription 1, TATA box binding protein binding
191907	BTF3	−0.32	−0.98	0.09	0.00	Basic transcription factor 3
48413	ZNF195	−0.66	−1.05	−0.30	−0.05	Zinc finger protein 195
147106	BACH1	−0.75	−1.19	−0.31	−0.60	BTB and CNC homology 1, basic leucine zipper transcription factor 1
267094	HIVEP1	−0.72	−1.26	−0.25	−0.09	Human immunodeficiency virus type 1 enhancer binding protein1
196174	ATF7	−0.60	−1.26	−0.35	−0.44	Activating transcription factor 7
487820	ZFP36L2	−0.88	−1.27	−0.37	−0.26	Zinc finger protein 36, C3H type-like 2
501971	TCFL1	−0.37	−1.35	0.10	−0.01	Transcription factor-like 1
52681	RELB	−0.24	−1.48	−0.05	−0.10	v-Rel reticuloendotheliosis viral oncogene homolog B, nuclear factor-κ light polypeptide gene
310138	FOXF2	0.03	−1.85	−0.22	0.05	Forkhead box F_2
487113	MSX1	−0.94	−1.94	−0.37	−0.56	msh Homeo box homolog 1 (*Drosophila*)
182411	EGR1	−0.88	−2.09	−0.28	−0.68	Early growth response 1

genes of NF-κB: E-selectin, VCAM-1, PDGF, tissue factor (all SNP), IL-1B, and VEGF (both SNP and DETA-NONOate; see also web appendix www.nephrogenomics.net/data/appendices/ECexoNO-AJP2004). Furthermore, DETA-NONOate depressed both RELB and the p100 precursor of NF-κB 2 (p49). Although NF-κB has been implicated as an important player in the progression of vascular cell function (12), reports about RELB and the vasculature are scarse. Increased levels of RELB have been reported in a balloon catheter injury model in the rat carotid artery (24). Taken together, the data suggest a role for endothelial regulation of RELB, which warrants further investigation.

Inhibition of GC, one of the main targets of NO, prevented a large part of the transcriptional responses to NO. This suggests that at least part of the transmission is not dependent on sGC and the formation of cGMP. Evidence is rapidly accumulating for the hypothesis that NO can nitrosylate proteins with an alteration in function as a consequence and that

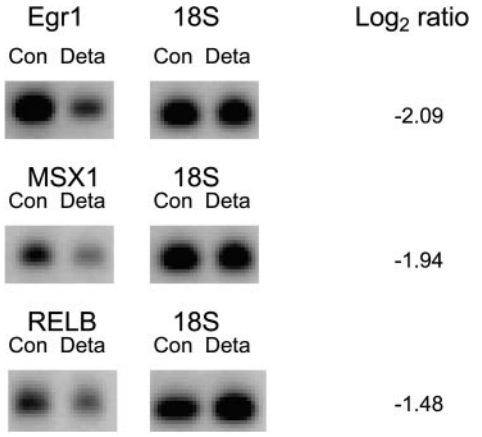

Fig. 6. RT-PCR of three of the four most pronouncedly depressed transcription factors upon exposure to 250 μM DETA-NONOate for 4 h. The average $\log_2$ ratio as obtained from the microarray analysis for each gene at 4-h exposure to DETA-NONOate is also depicted ($n = 4$ for each gene).

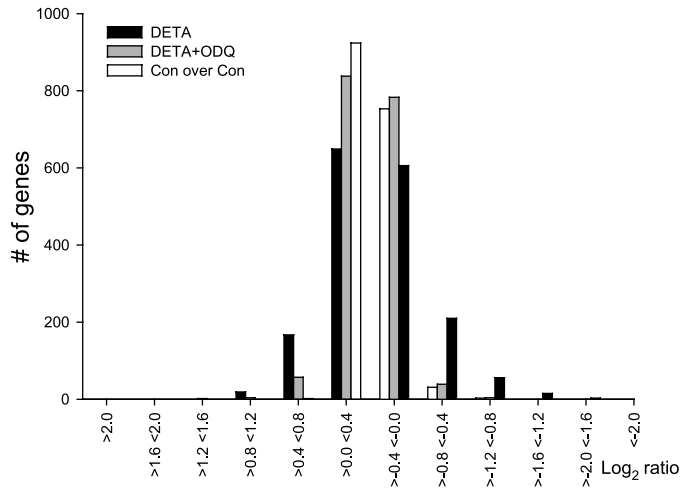

Fig. 7. Comparison of the distribution of the $\log_2$ ratios of HUVECs exposed to 250 μM DETA-NONOate for 4 h in the absence and presence of the guanylyl cyclase inhibitor ODQ. ODQ inhibited the changes of many, but not of all, genes that were affected by DETA-NONOate administration.

Table 3. *Gene expression responses to 250 μM DETA-NONOate in the presence and absence of the guanylate cyclase inhibitor ODQ*

Symbol	Accession No.	Description	DETA-NONOate	DETA-NONOate + ODQ
AP2B1	H12138	Adaptor-related protein complex 2, β1-subunit	−2.96	−0.14
PLP1	H29079	Proteolipid protein 1 (Pelizaeus-Merzbacher disease, spastic)	−1.89	−0.46
PTPRN2	AL547620	Protein tyrosine phosphatase, receptor type, N polypeptide	−1.81	−0.49
MVK	R59901	Mevalonate kinase (mevalonic aciduria)	−1.73	1.37
TYRP1	H86270	Tyrosinase-related protein 1	−1.49	−0.13
ASAH	BG430149	*N*-acylsphingosine amidohydrolase (acid ceramidase)	−1.46	−0.14
LMAN1	N30854	Lectin, mannose binding, 1	−1.45	−0.32
CCNG1	H15967	Cyclin G_1	−1.43	−1.17
ZNF195	H14414	Zinc finger protein 195	−1.34	0.23
VCAM1	H07071	Vascular cell adhesion molecule 1	−1.32	−0.54
GART	R59532	Phosphoribosylglycinamide formyltransferase, phosphoribose	−1.31	−0.11
OASIS	BG822562	Old astrocyte specifically induced substance	−1.24	−0.57
DSG1	W94221	Desmoglein 1	−1.22	−0.43
APC	H29191	Adenomatosis polyposis coli	−1.17	−0.20
COL6A1	R49887	Collagen, type VI, α_1	−1.14	−0.26
GRIK2	H15472	Glutamate receptor, ionotropic, kainate 2	−1.10	−0.81
ARG1	AA149501	Arginase, liver	−1.09	0.19
PDYN	R61564	Prodynorphin	−1.06	−0.51
PON2	AW966645	Paraoxonase 2	−1.04	0.01
TM4SF4	T83911	Transmembrane 4 superfamily member 4	−1.02	−0.16
RFC1	R54282	Replication factor C (activator 1) 1 (145 kDa)	−1.01	−0.31
UTRN	AA010522	Utrophin (homologous to dystrophin)	−1.00	−0.33
MAN2A2	N44492	Mannosidase-α, class 2A, member 2	1.02	0.64
AKR1C2	H64052	Aldo-keto reductase family 1, member C_2 (dihydrodiol dehydrogenase)	1.05	−0.51
APOB	W07144	Apolipoprotein B [including Ag(x) antigen]	1.12	0.68
DHCR24	W92108	24-Dehydrocholesterol reductase	1.35	0.81

ODQ, 1*H*-[1,2,4]oxadiazolo-[4,3-*a*]quinoxalin-1-one.

protein nitrosylation is a regulated process (39).The literature contains scattered data about cGMP-independent EC events including gene expression. Sata et al. (37) have demonstrated that NO inhibited apoptosis induced by serum deprivation of HUVECs and that this effect was not affected by soluble GC inhibition using ODQ. Support is available that this antiapoptotic effect of NO is mediated by nitrosylation of caspase-3 and is cGMP independent (35). Besides protein modification, NO can cause acute cGMP-independent increase in EC calcium levels (6). Taken together, we have found evidence that within the EC, NO can exert cGMP-independent influences on gene expression.

A global tendency of genes to react in the first 4 h and to fade after 8 h was observed, despite the additional supplementation of SNP to the medium during the experiment, because of the limited half-life of the compound. To ascertain that this was not related to the compound per se, the transcriptional responses to DETA-NONOate, which has a half-life of 20 h, were tested, and a similar time course of events was observed. Two mechanisms for desensitization have been described with respect to NO. Administration of NO results in acute (within minutes) desensitization of soluble GC, that is, cGMP increases and then stabilizes at a new plateau (5). This has been related to the activation of PDE 5 (28), which hydrolizes cGMP when phosphorylated. The other form of desensitization that has been suggested in via downregulation of GC (14). In a clinical setting, tolerance to nitrates, in particular to nitroglycerin, has been recognized decades ago (38). The mechanism remains largely unknown; a role for superoxide formation by NADPH oxidase (30) has been suggested. With respect to the present experiments, some aspects are interesting. First, the genomic tolerance is at the expressional level within the EC. Münzel et al. (29) have demonstrated that tolerance to exogenous nitrates of VSMCs is partly dependent on the presence of endothelium; here, we demonstrate that the tolerance is also a phenomenon intrinsic to the EC. Furthermore, the tolerance does not come from a decrease in cGMP formation. It has been suggested that prolonged exposure to NO leads to decreased soluble GC mRNA, either because of decreased transcription (14) or due to decreased stability of soluble GC mRNA (15). However, the present experiments indicate a decrease in genomic response to exogenous NO donors despite continuous presence of cGMP in ECs and the medium. This excludes the mentioned mechanisms and implies that cGMP-mediated signaling to the nucleus is dampened.

A possible explanation is formed by reported enhancement of DNA (cytosine-5-)-methyltransferase (DNA-MeTase) activity by NO (17). DNA-MeTase methylates CpG islands, which are present in the promoter regions of many genes. Such methylation favors a repressive chromatin structure that impedes promoter binding of transcriptional activators and leads to gene silencing (7). DNA-MeTase activity has been shown to be increased at a posttranscriptional level by the NO donor *S*-nitroso-*N*-acetyl penicillamine (SNAP) and to be responsible for silencing of fragile X mental retardation gene. Inhibition of DNA-MeTase using 5-aza-2′-deoxycytidine prevented this silencing (17). Irrespective of the mechanism, the desensitization of the transcriptional apparatus to cGMP could serve as a protective mechanism of the EC.

Some considerations should be mentioned regarding experimental design. The changes in gene expression of the nonstimulated ECs upon SNP exposure were relatively mild com-

pared with studies investigating transcriptional responses to exogenous NO in ECs after stimulation by hypoxia (1), IL-1α and -1β (11), LPS (32), or TNF-α (40). Considerable variation in the actions of different NO donors has been reported, such as the absence of stimulation of the VEGF promoter by SNP and SIN-1 and the strong induction by SNAP, GSNO, NOC18, and NOR4 of the same gene (22). These differences may be due to variation in the kinetics of NO release. DETA-NONOate, an NO donor with a very long half-life, showed a more prominent response, which made us use this compound to address the time course and the effects of concomitant soluble GC inhibition. GC is likely to be active in our EC culture, because we observed increased cGMP in the medium of the cells exposed to SNP and a functional response to inhibition of soluble GC. Similar functional responses have been obtained by others in primary cultures of ECs (16), suggesting that GC activity is present in HUVECs. Finally, the present study used cDNA microarrays to assess gene expression. These microarrays may underestimate the change in gene expression. Polacek et al. (34) recently reported that the accuracy of cDNA filter arrays to detect changes in gene expression is enhanced by first applying RNA amplification; thus the possibility exists that in the present study some changes may have remained unrecognized. It should be underlined that by and large, such drawbacks also exist for more conventional analysis as RT-PCR and Northern blot analysis. Along these lines, it has to be remarked that the present gene chip interrogated only a small part of the whole genome.

In summary, two different NO donors, SNP and DETA-NONOate, exerted a predominantly depressing effect on the expression of multiple genes, including a remarkable number of transcription factors, in human ECs in culture. The actions of NO were not maintained, despite the continued presence of elevated cGMP levels. Most, but not all, of these changes were prevented by inhibition of GC. This study demonstrates an early response in gene expression in ECs exposed to NO, which is largely but not exclusively mediated through cGMP, but quickly fades despite continued NO exposure. The background and physiological role of this secondary fading in response has to be elucidated. This resetting allows the cell to respond to increased shear without vigorous changes in gene expression and perhaps to prepare for further acute changes in shear.

ACKNOWLEDGMENTS

The assistance of Ele Holloway at the European Bioinformatics Institute with submission to ArrayExpress was greatly appreciated.

GRANTS

The research of B. Braam was supported by a fellowship of the Royal Dutch Academy of Arts and Sciences. Support for this study was also obtained from Dutch Kidney Foundation Grant NSN 6013.

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