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Journal of Chromatography B, 851 (2007) 3–11
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Review

Microarray-based gene expression profiling to elucidate cellular responses to nitric oxide—A review from an analytical and biomedical point of view☆

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Received 17 May 2006; accepted 14 July 2006
Available online 7 August 2006

Abstract

Nitric oxide (NO) produced by NO synthases (NOS) regulates a wide range of cellular functions. Analysis by gene arrays provides valuable information for identifying important elements of the cellular responses to NO. Such screening tools might be useful to elucidate NO-responsive regulators, which play a central role in mediating NO effects. Although the final importance of a particular gene is determined by the encoded protein and further protein modifications, measurements of RNA levels have proven to be partly valuable in identifying the molecular changes that occur in cells. Microarray technology permits large-scale and genome-wide analysis of gene expression from multiple samples. We review the current knowledge of the use of microarray gene expression screening in elucidating the effects of NO on various cells and tissues. We also point out the limitations of general microarray-based gene expression analyses and especially when investigating the effects of NO.

Keywords: Reviews; Microarray; Gene expression; Endothelium; Nitric oxide; Transcriptome; Sensitivity

Contents

☆ This paper is part of a special issue entitled "Analysis of the L-Arginine/NO pathway", guest edited by D. Tsikas.

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doi:10.1016/j.jchromb.2006.07.023

1. Introduction

Nitric oxide (NO) produced by various NO synthases (NOS) regulates a wide range of cellular functions, including maintenance of vascular tone, cellular migration, cell growth, differentiation and apoptosis (reviewed in [1] and references therein; [2]). Several isoforms of NOS exist; these are products of different genes, with different localization, regulation, catalytic properties and inhibitor sensitivity. Neuronal NOS (nNOS; also known as type I, NOS-I and NOS-1) is the isoform first found in neuronal tissue; inducible NOS (iNOS; also known as type II, NOS-II and NOS-2) is the isoform which is inducible in a wide range of cells and tissues, and lastly endothelial NOS (eNOS; also known as type III, NOS-III and NOS-3) is the isoform first found in vascular endothelial cells [3]. Two NOS monomers are usually associated with two molecules of calmodulin [1,4]. This complex additionally contains the tightly bound cofactors (6*R*)-5,6,7,8-tetrahydrobiopterin (BH_4), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and iron protoporphyrin IX (haem). NOS normally catalyse the conversion of L-arginine, nicotine adenine dinucleotide phosphate (NADPH), and oxygen to the free radical NO, L-citrulline and NADP [1,4]. In certain circumstances eNOS can become uncoupled and, in turn, produces superoxide anions instead of NO (e.g. in diabetes) [1]. Initial steps of NO-dependent signalling pathways involve nitrosation, nitration, or oxidation of specific targets such as soluble guanylyl cyclase, membrane associated G proteins and tyrosine kinases, resulting in induction of mitogen-activated protein (MAP) kinase cascades and activation of various transcription factors [3,4]. In general, most effects of NO are mediated by the second messenger cyclic 5′-guanosine monophosphate (cGMP), but cGMP-independent effects of NO on gene expression are additionally known [5,6].

Analysis by gene arrays provides valuable information in identifying important elements of the cellular responses to NO. Such screening tools might be useful to elucidate NO-responsive regulators, which play a central role in mediating NO effects. For instance, microarray analyses have shown that NO can act as an inducer or repressor of apoptosis, dependent on concentration and cell type. NO is able to prevent apoptosis in hepatocytes [7], but other cells such as peritoneal macrophages, neurons, pancreatic cells and thymocytes are rendered more sensitive to the induction of apoptosis by NO [8]. In the present review we summarize the currently available microarray studies which have investigated the effects of NO in cell cultures, NOS transgenic or knockout animals or animal models treated with NO donors. We also highlight the limitations and potential pitfalls of this methodology in the analysis of cellular responses to NO.

2. General aspects of microarray technology

Microarrays of DNA probes were introduced over 10 years ago with a subsequent rapid evolution of the technology. They allow rapid identification of altered expression of thousands of genes from tissue or cells obtained from different RNA sources, e.g. tissue from diseased versus healthy animals/patients or cells before and after treatment with various compounds. The process

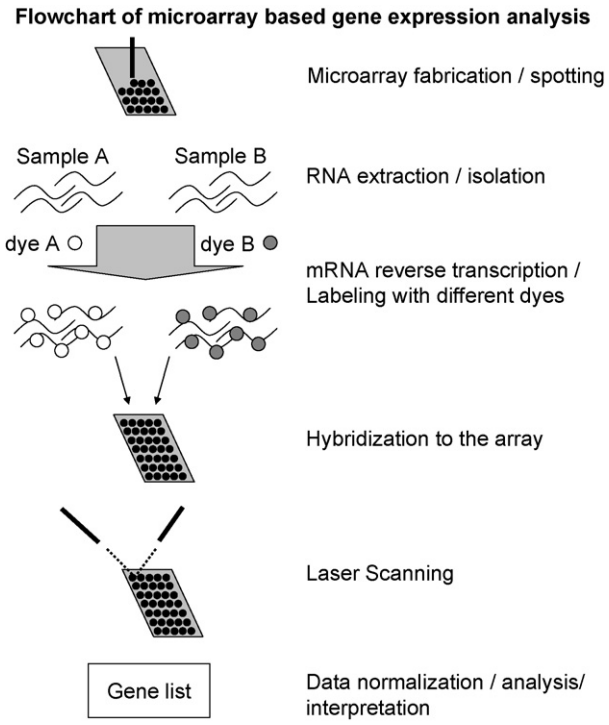

Fig. 1. A simplified flow-chart of microarray-based gene expression analysis. A brief description of the different steps of microarray analyses is given in Section 2.

of expression analysis can be broadly divided into three main steps, which include: (1) array fabrication; (2) probe preparation and hybridization; and (3) data collection, normalization and analysis.

The process of array fabrication has been recently reviewed in detail [9] and is not a present focus. Arrays can be spotted in-house using array spotters (see Section 4) or can be obtained from many manufacturers. Several aspects of the array fabrication process are covered in Section 4 of this review. In general, microarrays have DNA sequences on their surface, which can hybridize to complementary nucleotide sequences in a given sample. There are several variants of array DNA sequences that include short oligonucleotides (15–25 bp), long oligonucleutides (50–120 bp) and PCR-amplified cDNA clones immobilized to membranes or slides. A simplified flowchart of microarray-based gene expression analysis is given in Fig. 1. For gene expression analyses RNA must be isolated by appropriate methods, usually by phenol/chloroform-based methods, using the Trizol reagent (Invitrogen, Germany) or custom columns which bind RNA are used. An essential step is the analysis of RNA quality after isolation, for example, by the use of capillary electrophoresis [10]. Probes for microarray analysis are prepared from RNA templates by incorporation of fluorescent nucleotides during first strand cDNA synthesis (see Fig. 1) or by incorporation of tags which are later stained with fluorescent dyes. After the synthesis, unincorporated fluorescent nucleotides need to be removed by washing steps. Next, the sample is hybridized to the array. For this the sample is incubated together with hybridization buffers on the microarray for several hours manually or in special hybridization stations which can improve quality of the

Table 1
Effects of NO on a human monoblastoid cell line lacking the soluble guanylyl cyclase

Genbank number	Gene name	Description/function	Fold-change
U04810	TROAP	Cell adhesion	0.2
U22376	c-Myb	Cell cycle control	0.3
L47276	TOP2Ab	Spindle assembly	0.4
D14678	KIFCI	Spindle assembly	0.4
U14518	CENPA	Chromosome organization	0.4
U01038	PLK	Mitosis	0.4
M25753	CCNBI	Mitotic checkpoint	0.4
D38751	KIF22	Mitosis	0.4
U05340	CDC20	Ubiquitin-dependent protein degradation	0.5
M86699	TTK	Spindle assembly/mitotic checkpoint	0.5
U09579	CDKNIA (p21)	CDK inhibitor	2.1
J04111	c-jun	Regulation of cell cycle	3.7

Note: This table was constructed using the data from [14]. cGMP-independent effects of NO on gene expression in U937 cells exposed to the NO donor *S*-nitrosoglutathione (400 μM) vs. glutathione (400 μM) serving as control. Treatment duration was 6 h. Affymetrix HuGeneFL6800 microarrays were used, which contained >5000 unique transcripts. Data are given as fold-changes (see Section 2 for explanation).

hybridization by minimizing handling errors with the array. The incubation step is followed by a washing step to get rid off the non-incorporated probe. Finally, the array is scanned using laser light. In a next step, the grayscale images are generated from the arrays. From this step, analysis and interpretation of raw data needed to be performed.

For example, with cDNA arrays usually the ratio of two fluorescent dye intensities at any spotted gene represents the ratio of the corresponding mRNA molecules in the two samples. A first step in the process of data analysis is normalization of the obtained raw data or relative fluorescent intensities. This is important to adjust for differences in the labeling and detection efficiencies for the fluorescent labels and for differences in the quantity of starting RNA from various samples. Following normalization data are analyzed to identify genes which are differentially expressed. A popular concept is to use a post-normalization cut-off of twofold up- or down-regulation to define differential expression (fold-change). However, there is a considerable amount of different methods and approaches for microarray-based gene expression analysis available (for review see [11–13]).

3. Application of the microarray technology to study effects of NO in cell cultures and tissues

3.1. Effects of NO on mononuclear cells and fibroblasts

An initial microarray approach was chosen to address whether the soluble guanylyl cyclase is an important translator of the NO-mediated effects on cellular gene expression. In a human monoblastoid cell line which lacks the soluble guanylyl cyclase, NO regulated 110 transcripts, which mainly code for proteins involved in the regulation of cell cycle processes and cell proliferation [14]. These genes appear to be regulated independently of the soluble guanylyl cyclase. Details of regulated genes can be found in Table 1. Additionally, the effects of cyclic adenosine monophosphate (cAMP) on gene expression were studied and a comparison of NO- and cAMP-related effects demonstrated that NO regulation of cell cycle genes was independent of its ability to interfere with cAMP-signalling. Cell cycle genes induced by NO annotated to the GI/S phase of cell cycle and most of them were target genes of the transcription factor E2F involved in GI/S transition. E2F1 induces many G1/S phase genes including cyclin E1, cell division control protein 6 homolog (CDC6), uracil-DNA glycosylase, JUN, p21 and c-myb [15–22]. In contrast, repressed genes were associated to G2/M of which 8 out of 27 were known targets of p21. The known anti-proliferative effects of NO may rely to cGMP-independent mechanisms.

In a further study the expression profiles of NO-regulated genes in lymphoblastoid U937 cells and Mono Mac 6 monocytes were studied [23]. The NO chemical donor dipropylenetriamine NONOate (DPTA-NO) was used for the incubation studies at a final concentration of 500 μM. The authors identified 17 NO-dependent genes, which are involved in intercellular proinflammatory communication, cell signalling, cell cycle, apoptosis or gene expression (see Table 2 for details). Notably, the change of expression was time-dependent, as only 5 out of 17 genes retained substantial alterations of their expression 14 h after DPTA-NO exposure. Most genes upregulated in those cells encoded for transcription factors. Interestingly, the transcription factors c-fos and c-jun form the dimeric factor AP-1, which is a major activator of genes related to cell proliferation and stress response [24]. Additional transcription factors known to be regulated at least in part by NO are hypoxia inducible factor 1 (HIF-1), NFkappaB and iron regulatory proteins (IRPs) [25–27]. A second class of NO-induced genes in the study of Turpaev et al. encode for cell cycle regulators, such as cyclin-dependent kinases [23]. For instance, DPTA-NO treatment for 14 h led to a strong increase in expression of cyclin A2, which is a positive regulator of cyclin-dependent kinase 1 (CDK1) and CDK2. Likewise, the gene encoding for the CDK2 inhibitor p21/Cip1 was induced by NO. NO affected expression of genes encoding for intercellular communications, such as cytokines and chemokines. Expression of the inflammatory cytokine IL-8 and the vascular endothelial growth factor (VEGF) was highly induced by NO. Addition of the NO-scavenger 2-phenyl-4,4,5,5-tetramethyl-imidazole-1-oxyl 3-oxide (PTIO) attenuated increased expression of NO-regulated genes such as

Table 2
Deregulated genes in human monocytic cells exposed to NO

Accession number	Gene name	Description/function	Fold-change
AA001329	cyclin A2	Cell cycle positive regulator	0.4
H89939	CKS1	Cell cycle positive regulator	0.4
H58486	STK6	Cell cycle positive regulator	0.4
R17728	molybdopterin synthase	Sulfur metabolism	0.4
T84975	TRAF4-associated factor 1	Signal transduction	0.5
N76562	H-ferritin	Iron homeostasis	5.0
N91060	VEGF	Growth Factor	5.2
W52394	ATF-4	Transcription factor	5.3
AF347004	ND4	Oxidative phosphorylation	5.4
AA047110	PBEF	Cytokine	5.5
W52922	c-maf	Transcription factor	5.8
AF347004	ATPase 8	Oxidative phosphorylation	6.1
AF153609	SGK-1	Signal transduction	6.2
R07513	MIP-1-alpha	Cytokine	8.6
H83378	p21/Cip1	Cell cycle negative regulator	10.0
W33116	c-jun	Transcription factor	12.2
H29136	MKP1	Signal transduction	14.4
AA019816	c-fos	Transcription factor	14.6
W40425	IL-8	Cytokine	32.2

Note: This table was constructed using the data from [23]. Effects on gene expression pattern of NO-dependent genes in U937 and Mono Mac 6 monocytes exposed to the NO donor dipropylenetriamine NONOate (DPTA-NO) at 500 μM for 4 h. cDNA microarray slides from the Ontario Cancer Institute Microarray Center (Toronto, Canada) were used. These arrays contained 19,008 cDNA sequences encompassing both known genes and expressed sequence tags. Data are given as fold-changes.

c-jun and c-fos, whereas others were unchanged (e.g. c-maf, ferritin) or dramatically increased (e.g. IL-8). This dramatic increase may be explained by the fact that PTIO was added to the NO donor under conditions where it promoted N_2O_3 generation thus mimicking nitrosative stress [23].

An additional characterization of apoptotic-signalling pathways activated by NO in human lymphoblastoid cells was shown by Li et al. using cDNA microarray expression and immunoblotting [28]. The authors found a p53-mediated transcriptional response to NO in p53-wild type TK6 cells, but not in closely related p53-mutant WTK1 cells. NO led to an increase in previously known p53 target genes, such as phosphatase PM1D, oxidoreductase homolog PIG3 and NOXA. Additionally, NO modulated levels of several gene products in mitochondrial- and death-receptor-mediated pathways. These pathways were not induced in the presence of mutant p53, underlining the role of p53 in NO-mediated apoptotic effects.

In cultured mononuclear cells, NO regulated a diverse subset of genes involved in inflammation, proliferation, metabolism, apoptosis, cell cycle, signal transduction and transport. Cell cycle seems to be a major target of NO-mediated gene regulation at least in mononuclear cells. The sets of NO-sensitive genes reported in the studies of Cui et al. [14] and Turpaev et al. [23] seem to be specific for monocytic cell lines, as others have found totally different gene sets altered in response to NO when other tissues or cell lines were investigated [29]. Using a cDNA microarray approach Yook et al. have shown that the NO donor *S*-nitroso-*N*-acetyl-penicillamine (SNAP, 100 μM) strongly induces Bcl-2/adenovirus E1B 19kDa-interacting protein 3 (BNIP3) in cultured macrophages. In contrast, macrophages from NOS-2-null mice failed to produce BNIP3 in response to lipopolysaccharide underlining the role of NO in this context [30]. Mitsumoto et al. used microarray analysis to recognize the dynamic molecular changes of human fibroblasts exposed to NO [31]. Among over 300 NO-sensitive genes, the authors identified a gene coding for the protein transforming growth factor-beta inducible early response 1 (TIEG1), that plays a key role in TGF-beta regulated cell growth control and apoptosis. This pathway may have important roles in various molecular decision pathways in cells that sustain inflammatory damage under pathophysiological conditions.

3.2. *Effects of NO on cardiovascular cells*

Changes of gene expression in pulmonary artery endothelial cells from pigs where studied after addition of the NO donor (*Z*)-1-[2-(2-Aminoethyl)-*N*-(2-ammonio-ethyl)amino]diazen-1-ium-1,2-diolate (NOC-18, 1 mM) [32]. It has been reported that NO released from 1 mM NOC-18 results in steady state levels of 1–3 μM NO in medium without any cofactors (see [33] and product technique data of Calbiochem, Germany). This would be comparable to endogenous concentrations generated by iNOS after cytokine stimulation [34]. NO increased a group of genes encoding for proteins involved in apoptosis, such as Bcl-xl, inhibitor of apoptosis protein-1 (IAP-1), IAP-2 and growth arrest and DNA damage-inducible protein 45 (GADD45). Additionally, NO regulated expression of genes implicated in a variety of signal transduction pathways, including the Jak/Src, hedgehog, NFkappaB and p53 pathway, which regulate critical cellular functions such as apoptosis, cell growth and DNA damage/repair. Another study used microarray techniques to assess the impact of NO on the expression of phosphodiesterase (PDE) genes in rat pulmonary arterial smooth muscle cells [35]. Incubation of cells with *S*-nitroso-L-glutathione (GSNO) increased expression of PDE4B, a PDE

Table 3
Effects of NO on rat aorta

Ascession number	Gene name	Description/function	Fold-change
AA818412	Cytochrome p-450, phenobarbital-inducible	Metabolism	0.5
Al030295	GTP cyclohydrolase I	Metabolism	0.5
AA924618	High molecular weight K-kininogen	Metabolism	0.5
AA925580	Stannin	Growth control	0.5
AA858694	Thyroid nuclear factor 1	Transcription factor	0.5
AA818082	myr5	Small GTPase	0.5
AA964044	Apolipoprotein A-l (apoA-l)	Metabolism	0.5
AA817928	thy-1 antigen	T-cell antigen	0.5
AA901070	Cpi-26	Protease	0.5
AA859471	7-Dehydrocholesterol reductase	Metabolism	0.5
Al144995	Na^+/bicarbonate cotransporter	Transporter	2.0
Al071947	Cortactin-binding protein 1	Unknown	2.0
Al071529	p27	Cell cycle control	2.1
AI045179	STAT3	Transcription factor	2.2
AA956438	Mxi1	Cell cycle control	2.2
AA956238	Vitronectin	Cell structure	2.2
AI072547	Tyrosine phosphatase CBPTP	Cell growth	2.3

Note: This table was constructed using data from [39]. Vascular gene expression after treatment of rats with 10 μg/min nitroglycerine for 8 h. Rat GF300 GENE FILTER Microarrays were used. These arrays consisted of 5147 spotted cDNAs with additional 384 spots containing genomic DNA and housekeeping genes. Data are given as fold-changes.

isoform that specifically metabolizes cAMP. Mechanistically, induction of PDE4B by NO requires cGMP synthesis by the soluble guanylyl cyclase. This study demonstrated that NO increased expression of a cAMP-specific PDE that may provide evidence for a potential cross-talk between cGMP- and cAMP-signalling pathways.

Gene expression profiles are also deregulated in various cardiovascular diseases, such as end-stage heart failure [36,37]. Unloading of the failing heart with a left ventricular assist device may normalize exaggerated gene expression and cardiac function [38]. Using microarray profiling, Chen et al. found 130 gene transcripts to be increased and 49 to be downregulated after unloading of failing hearts with assist devices [38]. Upregulated genes included transcription factors, genes related to cell growth/apoptosis/DNA repair, cell structure proteins, metabolism and cell signalling. Interestingly, genes coding for eNOS and the dimethylarginine dimethylaminohydrolase (DDAH) isoform I, which regulates partly endogenous eNOS activity, were significantly increased after unloading of the failing hearts by implantation of assist devices. This highlights a role for genes involved in NO-signalling in heart failure.

In rat aortic tissue, cDNA microarray analyses were performed after infusion of animals with the two NO donors nitroglycerin (NTG) and SNAP [39]. Continuous NTG infusion (rate 10 μg/min for both NTG or SNAP) resulted in widespread changes in vascular gene expression, many of which are consistent with the multifactorial and complex mechanisms reported for nitrate tolerance (for gene expression details see Table 3). This was not seen for SNAP and corresponds nicely to an in vivo study that likewise did not found any nitrate tolerance after infusion of SNAP in the rabbit [40]. Obviously, pharmacological actions of NO donors are not identical especially when alterations in gene expression profiles are investigated. This may be, at least in part, due to the finding that most organic nitrates additionally lead to the secondary production of superoxide in addition to their metabolism to NO and therefore in turn may regulate additionally stress-sensitive genes (see [1] and Section 4.2 of this article).

3.3. Effects of NO on the brain

To study the action of NO in the brain, a transgenic mouse model was used that expressed nNOS under the control of the promoter of the neuron-specific calcium-calmodulin multifunctional kinase II alpha gene [41]. Using gene expression profiling assays the authors demonstrated NO-mediated alterations of genes coding for proteins involved especially in cell division and gene activity.

3.4. Effects of NO on adipose tissue

The amino acid L-arginine is the physiologic precursor of NO. Fu et al. investigated changes of gene expression after L-arginine supplementation in fat tissue to test whether NO formation by L-arginine treatment would reduce fat mass in diabetic rats and to analyze underlying molecular mechanisms [42]. L-Arginine administration (1.25% in drinking water for up to 10 weeks) increased serum concentrations of NO metabolites by 70% and body weight was decreased 4–10 weeks after L-arginine supplementation. Microarray analysis indicated that L-arginine supplementation increased key genes responsible for fatty acid and glucose oxidation in adipose tissue (e.g. NOS-1, heme-oxygenase-3, AMP-activated kinase, peroxisome proliferator-activated receptor gamma coactivator-1alpha). This may explain the fat-reducing effects of L-arginine treatment.

3.5. Effects of NO on the liver

Liu et al. tested the effects of the liver-selective NO donor O2-vinyl 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate on

acetaminophen-induced hepatotoxicity in mice [43]. This NO donor is activated by cytochrome P450 enzymes to release NO especially in the liver. Genomic analysis of the livers after acetaminophen intoxication showed enhanced expression of genes associated with oxidative stress, apoptosis/cell death and DNA damage/repair. These alterations were significantly attenuated by co-treatment with the NO donor. This protection may involve reduction of oxidative stress, inhibition of apoptosis and the maintenance of hepatic vasculature to prevent congestion. Further support comes from the study of Zamora et al. who used a DNA microarray technique to determine alterations in mouse hepatocytes exposed to NO [44]. The authors report gene array data from isolated mRNA derived from iNOS-null mouse hepatocytes harvested from mice exposed to NO by infection with an adenovirus expressing human iNOS. A summary of deregulated genes is depicted in Table 4. About 200 genes were modulated, most of which encoded for proteins involved in inflammation, cell proliferation, cellular ener-

Table 4
Effects of human iNOS expression in hepatocytes from iNOS-null mice

Ascession number	Gene name	Description/function	Fold-change
AA105294	Elongation factor 2	tRNA transfer	0.1
AA109527	Actin 1	Cell structure	0.1
AA108110	Intiation factor 5A (EIF-5A) (EIF-4D)	Cell cycle control	0.1
AA087943	Actin, cytoplasmic beta	Cell structure	0.1
AA087943	Actin, cytoplasmatic A-X	Cell structure	0.1
X70298	Sox-4	Cell death	0.1
U34960	Mouse G protein beta 2 subunit mRNA	Signal transduction	0.1
U28728	Efs	Signal transduction	0.2
W16377	Myosin heavy chain	Cell structure	0.2
U37413	Mouse G alpha 11 subunit mRNA	Signal transduction	0.2
W77121	Homologous to sp P04574 (CANP)	Signal transduction	0.2
U65313	G3BP	Signal transduction	0.2
AA03109	Alpha-actin 1, cytoskelet isoform	Cell structure	0.2
AA120586	APO B-100/APO B-48	Lipid synthesis	0.2
W41501	R-ras	Cell death	0.2
W85447	Homologous to sp P30153 (PP2A), PR65-alpha	Signal transduction	0.2
D90173	NfiA	Signal transduction	0.2
D45850	Estradiol 17-beta-dehydrogenase	Lipid synthesis	0.3
W99875	Pyruvat kinase, M1	Metabolism	0.3
U08378	Stat3	Signal transduction	0.3
AA168633	von Willebrand factor precursor	Coagulation	0.5
AA064021	Homologous to sp P34884 MIF	Signal transduction	2.0
U17162	BAG-1	Cell death	2.1
U60530	Madr2	Signal transduction	2.9
W78338	Homologous to sp P25388	Unknown	3.0
V00836	Nerve growth factor beta (ß-NGF)	Growth factor	3.0
U77630	Adrenomedulin precursor mRNA	Stress response	3.1
W08033	Homologous to sp P06749	Signal transduction	3.6
U43900	STAM	Signal transduction	3.6
Z46663	Growth Hormone	Signal transduction	3.7
L40406	HSP-E71	Stress response	3.9
X75888	Cyclin E	Cell cycle control	4.2
Z46720	PICK1	Signal transduction	4.2
AA144400	PPASE	Stress response	4.2
W49178	Homologous to sp P31864	Unknown	4.3
U78103	EED	Cell cycle control	4.3
X74145	CRK 4	Cell cycle control	4.5
M21332	Mh + E22C class 111 RD gene	Inflammation	4.6
D88793	Mouse mRNA for cysteine rich protein-1	Cell growth	5.2
W51181	Homologous to sp P41584	Cell cycle control	5.2
AA106116	HSP70	Stress response	5.4
W55634	Dynactin	Cell structure	5.4
X57800	Polymerase delta auxiliary protein PCNA	Cell cycle control	5.9
D86726	Mouse mRNA for mMIS5	Cell cycle control	6.0
X56824	Mouse tumor induced 32-kDa protein	Unknown	6.2
X56824	Heme-oxygenase	Stress response	6.2
D86725	Mouse mRNA for mMCM2	Cell cycle control	6.7
U63337	Cdk2-alpha	Cell cycle control	9.3

Note: The table was constructed using data from [44]. Gene array analysis on mRNA from iNOS-null mouse hepatocytes harvested from mice exposed to NO by infection with an adenovirus expressing human iNOS construct that endogenously produces excessive NO. Analyses were performed 24 h after transfection. The Affymetrix Mu6500 GeneChip was used, which contained >6500 different transcripts. Data are given as fold-changes.

getics and, again, apoptosis. iNOS-derived NO appears to display an anti-inflammatory and anti-apoptotic role in hepatocytes, but also acts to suppress proliferation and protein synthesis. This is a good example of the apparent opposite effects of NO in different tissues. In monocytes NO led to a strong induction of c-fos and c-jun that form the dimeric transcription factor AP-1. In contrast, in the liver NO prevented the acetaminophen-induced induction of this transcription factor. Increased expression of apoptotic genes in hepatotoxicity was normalised by tissue-specific NO formation, whereas in mononuclear cells NO exerts mainly pro-apoptotic effects [23].

3.6. Effects of NO on bacteria

Microarray analysis in bacteria also can be used to identify NO-regulated genes. Patients with cystic fibrosis show a high prevalence for infection with pseudomonas aeruginosa. Treatment of pseudomonas aeruginosa with GSNO led to a great up-regulation of many nitrosative defense genes, e.g. of the 30 most induced genes 12 coded for proteins involved in metabolizing oxides of nitrogen [45]. NO is a potently bactericidal component of the innate immune system and has been used for inhalation therapies to treat pseudomonas aeruginosa infections of the lung. The present microarray data [45] may have clinical relevance and may explain the lack of effect of clinical NO therapies of patients with cystic fibrosis due to induction of NO-metabolizing, nitrosative defense systems.

4. Analytical aspects and limitations of microarray-based gene expression analyses

4.1. Analytical aspects and limitations of microarray-based gene expression analyses

The performance of microarrays can be measured by many parameters and has various confounding factors. Spotted microarrays are produced either by contact printing that is based on high definition pins which deposit the probe solution upon contact with the coated microarray substrate. Usually, the pins are attached to a robotic arm that moves the pins between the different probe solutions, the glass slides, where the microarray is created and a washing station. In contrast, non-contact printing uses small dispensing systems instead of pins, such as inkjet, bubble-jet or piezo actuation technology. The dispensing system usually dispense in the range of 100 pl to 2 μl to the array. Contact printing usually results in spot densities of 1500–5000 spots/cm^2 while non-contact printing can have slightly higher spot densities. There are several other possibilities to increase the spot density (reviewed in [9]). However, various problems arise with subsequent miniaturisation of microarrays. With a decrease in spot-size the resolution of the scanners must be improved. In turn, fluorescent scanners with higher resolution had been developed in the past. As of today the minimum resolution is about 2.5–5 μm that would allow to detect spot densities of about 30,000/cm^2 assuming 30 μm between each spot [9]. A further problem of decreasing spot-sizes is that the number of probe molecules can be reduced in a way that the dynamic range of a spot may be impeded [46]. Of further importance is a well-defined geometry of the microarray meaning that each spot should be at exactly the same distance from each other within an array (spatial localization of spots in the microarray). This is important as in a next step the scanned array requires a so-called grid overlaying the graphic image. The grid is determined by a computer and is needed to correlate a given spot with the spot position for subsequent quantitative analysis. Often laborious manual adjustment of the grid is needed when the spot geometry was not perfect during the spotting process. Other problems could arise during the spotting process such as production of incomplete or lacking spots due to a failure of delivering the drops from the printing tool to the surface during printing.

There are also differences between contact and non-contact printing procedures. Indeed, a significant better spot morphology has been observed on hydrophobic surfaces using non-contact printing compared to contact printing [47]. Factors that affect the spot morphology are for instance the spotting buffer, temperature and humidity. To improve reproducibility of the results it is mandatory that a variety of replicates (minimum 3–4) of a given DNA should be spotted on the microarray. The sensitivity threshold of microarray measurements defines the concentration range in which accurate measurements can be made (for review see [48]). There are some studies available which compared results from microarray-based gene expression analyses with quantitative PCR methods. Dependent on the microarrays and techniques used, the detection limit of the microarrays was in the range of two [49] to ten [50] gene copies per cell. However, the microarrays failed to produce meaningful measurements below that threshold. Although the sensitivity is impressive, it might still be insufficient to detect relevant changes in low abundance genes. Assessing the accuracy of microarray measurements requires that true concentrations of genes are available for a number of transcripts. True concentrations can be obtained by either spike-in or dilution experiments [51] or by measuring transcript levels with other methods such as quantitative RT–PCR or northern blots. In a study of Choe et al. Affymetrix GeneChip data for *Drosophila* RNA samples with about 1300 spiked-in genes were compared against a defined background of about 2500 genes [52]. The results suggested that the detection of about 70% of true positives can be achieved before reaching a 10% false-discovery rate. However, experiments demonstrate that microarray measurements may not be reliable for genes expressed at low levels and therefore at least a second quantification method, such as quantitative real-time PCR should be used for differentially expressed low abundance genes before planning further experiments.

Owing to the great amount of confounding factors, there has been a consensus that microarray studies should only be published when a Minimum Information About a Microarray Experiment (MIAME) is available. These MIAME criteria describe the minimum information required to ensure that microarray data can be easily interpreted and that results derived from its analysis can be independently verified. A final goal would be to establish a standard for recording and

reporting microarray-based gene expression data, which will in turn facilitate the establishment of databases and public repositories and enable the development of data analysis tools [53].

4.2. Pitfalls and limitations in the determination of NO effects by microarray analysis

A major drawback in elucidating cellular responses to NO using microarray data is that the gene expression does not necessarily translate to protein expression and/or function. Therefore, it only can be used as a screening tool and subsequent in depth molecular analyses have to be performed. Further, this technique cannot be used to clarify whether alterations of gene expression in response to NO donors or over-expressed NOS isoforms can be directly attributed to NO (NO-sensitive genes), or whether this is the consequence of changes of other NO-regulated genes (NO-insensitive genes).

Furthermore, a variety of experiments were performed with organic nitrates as NO donors under the assumption that NO is the only molecule that is formed (see for example [35]). However, in addition to NO donation most nitrates may lead to enhanced formation of superoxide, induce endothelial dysfunction, and impair vascular NO/cGMP signalling (reviewed in [1]). Thus, not only NO-sensitive genes are regulated after treatment with nitrates such as NTG, but also genes sensitive for reactive oxygen species (ROS). This explains at least in part the different cellular responses to different NO (and/or ROS) donors investigated in the past. Gene expression analysis using microarray techniques only represent a snap-shot of the cellular responses to NO. As a consequence, for a proper assessment of NO-regulated genes dose- and time-responses to the used NO donors are needed. In fact, the high costs of microarrays limit this approach. An alternative would be a more focused analysis of genes that are strongly regulated in the microarray using cheaper methods such as RT-PCR. Important events after the release of NO, such as nitrosation, nitration, or oxidation of specific targets resulting in activation of various transcription factors leading to changes in the expression of NO-regulated genes are not detected by microarray analyses, but are of equal importance for understanding NO-mediated effects. It should be pointed out that in many experiments that investigate the biological effects of NO donors exaggerated high levels are used that may limit the correlation of the results with real NO concentrations in healthy or diseases tissues. This holds true especially for *S*-nitrosothiols such as GSNO or SNAP, which are poor NO donors but be rather efficient in *S*-transnitrosylation of various proteins [54]. It turn it is not clear whether any observed effect is mediated by NO or *S*-transnitrosylation of certain proteins.

5. Conclusions

The microarray technology permits large-scale and genome-wide analysis of gene expression from different samples. In its appropriate sensitivity range, the existence and direction of gene expression changes can be reliably detected for the majority of genes. However, detection of low abundance genes are currently beyond the reach of microarray technology. An important finding of the analysis of global gene expression data is the fact that NO activates different gene networks in different tissues. Microarray-based gene expression screening is a valuable starting tool to determine the diverse function of NO in various tissues. However, many limitations and major drawbacks as described above should be taken into account from planning the experiments to interpretation of the results. Therefore, this technique should only be used in conjunction with other molecular and biochemical methods to elucidate the cellular responses of cells and tissues to NO.

Nomenclature

BH_4	(6*R*)-5,6,7,8-tetrahydrobiopterin
BNIP3	Bcl-2/adenovirus E1B 19kDa-interacting protein 3
cAMP	cyclic adenosine monophosphate
cGMP	cyclic 5′-guanosine monophosphate cyclin-dependent kinase (CDK)
DDAH	dimethylarginine dimethylaminohydrolase
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
GADD	growth arrest and DNA damage-inducible protein
GSNO	*S*-nitroso-L-glutathione
IAP	inhibitor of apoptosis protein
IRPs	iron regulatory proteins
MAP	mitogen-activated protein
NADPH	nicotine adenine dinucleotide phosphate
NOC-18	(*Z*)-1-[2-(2-Aminoethyl)-*N*-(2-ammonio-ethyl)amino]diazen-1-ium-1,2-diolate
NOS	NO synthases
NTG	nitroglycerin
PDE	phosphodiesterase
PTIO	2-(4-carboxyphenyl)-4,4,5,5-tetramethy-limidazoline-1-oxyl 3-oxide
SNAP	*S*-nitroso-*N*-acetyl-penicillamine
TIEG1	transforming growth factor-beta inducible early response 1
VEGF	vascular endothelial growth factor

Acknowledgements

This work was supported in part by grants of the *IZKF-Nachwuchsgruppe Cardiac Wounding and Healing* (E-31 to TT), *the Ernst und Berta Grimmke-Stiftung* (to TT), *and the NOVARTIS-STIFTUNG FÜR THERAPEUTISCHE FORSCHUNG* (to TT and JB).

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