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cDNA Microarray Analysis of Vascular Gene Expression After Nitric Oxide Donor Infusions in Rats: Implications for Nitrate Tolerance Mechanisms

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ABSTRACT Vascular nitrate tolerance is often accompanied by changes in the activity and/or expression of a number of proteins. However, it is not known whether these changes are associated with the vasodilatory properties of nitrates, or with their tolerance mechanisms. We examined the hemodynamic effects and vascular gene expressions of 2 nitric oxide (NO) donors: nitroglycerin (NTG) and S-nitroso-N-acetylpenicillamine (SNAP). Rats received 10 µg/min NTG, SNAP, or vehicle infusion for 8 hours. Hemodynamic tolerance was monitored by the maximal mean arterial pressure (MAP) response to a 30-µg NTG or SNAP bolus challenge dose (CD) at various times during infusion. Gene expression in rat aorta after NTG or SNAP treatment was determined using cDNA microarrays, and the relative differences in expression after drug treatment were evaluated using several statistical techniques. MAP response of the NTG CD was attenuated from the first hour of NTG infusion ($P < .001$, analysis of variance [ANOVA]), but not after SNAP ($P > .05$, ANOVA) or control infusion ($P > .05$, ANOVA).Student *t*-statistics revealed that 447 rat genes in the aorta were significantly altered by NTG treatment ($P < .05$). An adjusted *t*-statistic approach using resampling techniques identified a subset of 290 genes that remained significantly different between NTG treatment vs control. In contrast, SNAP treatment resulted in the up-regulation of only 7 genes and the down-regulation of 34 genes. These results indicate that continuous NTG infusion induced widespread changes in vascular gene expression, many of which are consistent with the multifactorial and complex mechanisms reported for nitrate tolerance.

KEYWORDS: DNA microarray, gene regulation, nitrate tolerance, nitric oxide donor, nitroglycerin.

INTRODUCTION

Since the identification of nitric oxide (NO) as an endothelium-derived relaxing factor, [1,2] various NO donors have been used for exploring the mechanisms of NO action. The NO donors used have originated from various chemical classes, including organic nitrates, S-nitrosothiols, sydnonimines, and sodium nitroprusside. Although all NO donors release NO, they may exert dissimilar pharmacological responses because of possible differences in the redox species of NO produced, tissue distribution, and susceptibility toward metabolic activation.[3,4]

Nitroglycerin (NTG), a representative organic nitrate, was first introduced in the late 1800s for the treatment of angina pectoris. While this NO donor is still widely used in cardiovascular therapy today, its long-term clinical usefulness is limited by the development of pharmacological tolerance, which was observed for all organic nitrates, regardless of the dosage forms.[5] The mechanisms of vascular nitrate tolerance are believed to be multifactorial, including decreases in intracellular thiol levels[6]; reductions in the activity of NTG metabolizing enzymes[7] and cyclic guanosine 3',5'-monophosphate (cGMP) production[8]; increased oxidative stress[9]; and changes in the expression/activity of endothelin-1, protein kinase C, [10] or endothelial NO synthase.[11] The presence of these widespread, and seemingly unconnected, alterations would suggest the possibility of additional regulatory changes that have yet to be identified. Recent advances in DNA microarray technology have enabled investigators to monitor gene expression on a large scale.[12] A typical high-density microarray contains thousands of genes spotted or immobilized on the matrix. This novel technique offers a significant advantage in terms of the number of genes that can be simultaneously analyzed, compared to conventional methods such as Northern blot analysis and reverse transcription-polymerase chain reaction (RT-PCR). DNA microarrays have been reported to provide quantitative data comparable to Northern blot analysis in general.[13] It appears attractive, therefore, to employ this newly developed technology for exploring the scope of regulatory changes in the vasculature as a result of nitrate tolerance.

In these studies, we used S-nitroso-N-acetylpenicillamine (SNAP) as a negative NO donor control of vascular tolerance. SNAP is a member of S-nitrosothiols (RSNO), a class of NO donors that have been proposed to serve as endogenous carriers of NO in the circulation.[14,15] Importantly, several RSNOs, including SNAP[16,17] and S-nitrosocaptopril,[18] have been reported to produce little or no pharmacological tolerance both in vitro and in vivo. Thus, this class of NO donor can be conveniently

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used as a negative control for the examination of the mechanisms of nitrate tolerance.

In this investigation, we therefore explored the use of gene microarray technology to compare and contrast the scopes of changes in vascular gene expression after continuous infusions of NTG, SNAP, or control vehicle in conscious rats, and to examine whether these changes may be consistent with the various existing mechanisms of vascular nitrate tolerance.

MATERIALS AND METHODS

Materials

NTG solution (1 mg/mL in 5% dextrose, D5W) was obtained from Schwarz Pharma (Monheim, Germany). SNAP was purchased from Alexis Corp (San Diego, CA), and prepared in D5W. The "Perfect RNA™ Eukaryotic Minikit" for RNA isolation was obtained from Eppendorf (Westbury, NY). ^{33}P dCTP was obtained form Amersham Pharmacia Biotech Inc (Piscataway, NJ). Rat GF300 GENEFILTERS® microarrays and other reagents were purchased from Research Genetics Inc (Huntsville, AL).

Animal Surgery

All surgical procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee of the University at Buffalo. Male Sprague-Dawley rats weighing 300-400 grams were obtained from Harlan (Indianapolis, IN). Two days prior to the in vivo hemodynamic study, 3 catheters were implanted in animals at the following sites: the left femoral artery for blood pressure measurements, the left femoral vein for bolus drug administration, and the right jugular vein for drug infusion.

In Vivo Hemodynamic Studies

Systolic and diastolic blood pressures were recorded continuously using a Statham pressure transducer (Ohmeda Inc, Murray Hill, NJ) and a Gould RS3400 recorder (Gould Inc, Cleveland, OH). Baseline blood pressure was allowed to stabilize for at least 15-30 minutes before starting the experiment. To document the presence of NTG tolerance, maximum mean arterial pressure (MAP) response to a 30-µg NTG intravenous (IV) bolus challenge dose (CD) was determined. Rats then received continuous infusion of 10 µg/min NTG, SNAP, or D5W vehicle for 8 hours (n = 4-6 animals for each infusion group). Maximal MAP response to the hourly NTG CD was measured, and compared to the response obtained prior to drug infusion. To determine the presence of self-tolerance, SNAP-infused animals also received a 30-µg SNAP bolus dose at baseline and every 2 hours thereafter. SNAP bolus CD was administered 15 minutes after the NTG CD. The infusion dose of 10 µg/min was chosen because previous studies of in vivo tolerance of NTG and SNAP in rats with congestive heart failure used this dose.[17] The use of a 30-µg bolus of NTG as a challenge dose was based on other studies (unpublished data) showing that this dose produced significant, rapid, and reversible hypotensive effects in conscious rats.

Total RNA Isolation

In separate studies, rats were infused continuously with 10 µg/min NTG or D5W vehicle (n = 4 each) for 8 hours via the right jugular vein. At the end of the infusion, the thoracic aorta was isolated and snap-frozen in liquid nitrogen. Total RNA from the rat aorta was isolated using the Perfect RNA Eukaryotic Minikit according to protocols recommended by the manufacturer. RNA concentration in the aortic sample was determined via spectrophotometry by measuring absorbance at 260 nm. RNA samples were stored at -80°C until the microarray assay. An identical experiment was conducted using SNAP vs vehicle control.

cDNA Microarray

Rat GF300 GENE FILTERS microarrays were treated according to the protocols established by the manufacturer. The GF300 microarrays consisted of 5 147 cDNAs with an additional 384 spots containing genomic DNA and housekeeping genes (β-actin). Microarrays were pre-hybridized with a hybridization solution containing COT-1 DNA (1 µg/mL) and poly-dA (1 µg/mL) in a hybridization roller oven (Biometra, Solon, OH) for 4 hours at 42°C. An aortic sample containing 3 µg of total RNA was converted to cDNA via reverse transcription with oligo-dT primers, and labeled with 33 dCTP. The labeling reaction was carried out at 37°C for 90 minutes, followed by purification of the labeled probe using a Bio-Spin 6 chromatography column (Bio-Rad, Hercules, CA). The probe was then denatured and hybridized with GENEFILTERS® microarray overnight (~18 hrs) at 42°C. GENEFILTERS® microarrays were washed as recommended in the protocol and then exposed overnight to a phosphor imaging screen and the signals were detected using a Cyclone PhosphorImager (Packard Instruments, Meridien, CT) equipped with OptiQuant analysis software (Packard Instruments, Meridien, CT). The microarray image was then imported to Pathways™, an array analysis software program (Research Genetics Inc, Huntsville, AL), and aligned using the control points on the GENEFILTERS microarray. The intensity of each spot on the array was processed and identified by the Pathways internal database. To remove systematic variations due to differences in RNA preparation and labeling efficiencies among samples, the raw intensity values were normalized by dividing each value by the

average intensity of all spots on an array (termed global or slide-wise normalization)[19]. In addition, 2 different sets of microarrays were used in each study and 4 replicates were carried for both treatment and control. This level of replication exceeded the recommendation for microarray experiments.[20] Microarray filters were stripped multiple times for reuse. The stripping efficiency of each microarray was checked by reexposing the microarray to the phosphor imaging screen and the signals were detected using the Cyclone PhosphorImager.

Microarray Data Analysis

To enable crossover determination on the microarray filters, 2 separate experiments were conducted, 1 involving NTG-treated (NTG1-NTG4) vs control (D5W1-D5W4), and another involving SNAP-treated (SNAP1-SNAP4) vs control (d5w1-d5w4). Differential gene expression between NO donor and D5W control was first evaluated using unpaired *t*-statistics. In order to assess the false discovery rate, that is, the proportion of falsely significant genes due to multiple statistical tests, a nonparametric approach based on resampling techniques was applied to the gene expression data. The resampling method refers to a statistical approach that constructs all possible outcomes within the same empirical data set via repeated sampling.[21,22] The observed test statistic (from the "true" grouping) is then compared against the distribution of test statistics from all possible data sets that are randomly generated.[21,22] This approach is widely accepted as a method to assess the reliability of reconstructed phylogenetic trees,[23] population genetics,[24] and biomedical experiments.[25] More recently, the use of the resampling-based methods (or bootstrapping) has been expanded to many other areas including the analysis of DNA microarray data.[26,27]

In this study, there existed 70 possible permutations of the expression data using 4 replicates for each treatment group ($8!/4!*4! = 70$). However, the *t*-statistics from 35 unique permutations of the data sets were calculated since the remaining 35 permutations are equal in magnitude, but with a negative sign. The permutation of the expression data was carried out as shown in Table 1 and the changes in gene expression were identified as significant if the given *t*-statistic from the data set was the highest value against the *t*-statistic distribution from all possible permutated data sets. Data analysis was also carried out using a recently published method for gene-array analysis, called "Significance analysis of microarrays" (SAM).[28] This technique also uses permutations of the data sets in order to control the false discovery rate.

Other Statistical Analysis

All other data are presented as mean ± SD. Statistical analysis was performed, where appropriate, using the Student *t*-test, or one-way analysis of variance (ANOVA), followed by the Student-Newman-Keuls post-hoc test. Differences with $P < .05$ were considered statistically significant.

Table 1. Illustration of Permutation-adjusted *t*-statistic Approach Using the Expression Data of the Genes for Liver Glutathione S-transferase Ya Subunit (Clones pGTR112 and pGTB38). The Changes in Gene Expression were Called Significant if the *t*-statistic of a Given Data Set Gave the Highest Value When Compared Against the *t*-statistic Distribution of All 35 Possible Permutated Data Sets.

	Possible permutated data sets (n=35)								*t*-statistics
	D5W1	D5W2	D5W3	D5W4	NTG1	NTG2	NTG3	NTG4	
1	16549.7	14494.6	20321.1	21456.8	12845.9	9780.5	11965.6	3567.0	**3.273***
2	D5W1	NTG1	D5W3	D5W4	D5W2	NTG2	NTG3	NTG4	2.575
3	D5W1	NTG3	D5W3	D5W4	NTG1	NTG2	D5W2	NTG4	2.296
4	NTG1	D5W2	D5W3	D5W4	D5W1	NTG2	NTG3	NTG4	1.985
5	NTG3	D5W2	D5W3	D5W4	NTG1	NTG2	D5W1	NTG4	1.785
6	D5W1	NTG2	D5W3	D5W4	NTG1	D5W2	NTG3	NTG4	1.757
.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.
31	NTG1	NTG4	D5W3	D5W4	D5W1	NTG2	NTG3	D5W2	0.308
32	NTG3	NTG4	D5W3	D5W4	NTG1	NTG2	D5W1	D5W2	0.207
33	D5W1	D5W2	NTG4	D5W4	NTG1	NTG2	NTG3	D5W3	0.065
34	D5W1	D5W2	D5W3	NTG4	NTG1	NTG2	NTG3	D5W4	0.063
35	NTG2	NTG4	D5W3	D5W4	NTG1	D5W1	NTG3	D5W2	0.041

*The highest value against the *t*-statistic distribution of all possible permutated data sets.

RESULTS

Differences in Hemodynamic Tolerance Between NTG and SNAP

Figure 1 shows the effects of 10 µg/min NTG, SNAP, or vehicle infusion, as such, on MAP. In normal conscious rats, continuous infusion of D5W vehicle had no apparent effect on MAP, which remained stable between 110-125 mm Hg throughout the 8-hour infusion time ($P > .05$, ANOVA). The variability of these measurements within the study period was approximately 10%. In both NTG- and SNAP-infused animals, the MAP also remained fairly constant throughout the study period ($P > .05$, ANOVA). Although MAP decreased slightly over time in the SNAP-infused group, the results did not reach statistical significance ($P > .05$, ANOVA). These results indicated that infusion of these 2 NO donors, at 10 µg/min, produced no apparent hypotensive effects in normal conscious animals.

Figure 2 shows the effects of drug infusion on the hypotensive effects of the hourly 30-µg NTG CD. With vehicle infusion, the hourly NTG CD all produced similar maximal MAP response throughout the entire study period ($P > .05$, ANOVA), confirming that vehicle infusion did not lead to any diminution of effect during the study

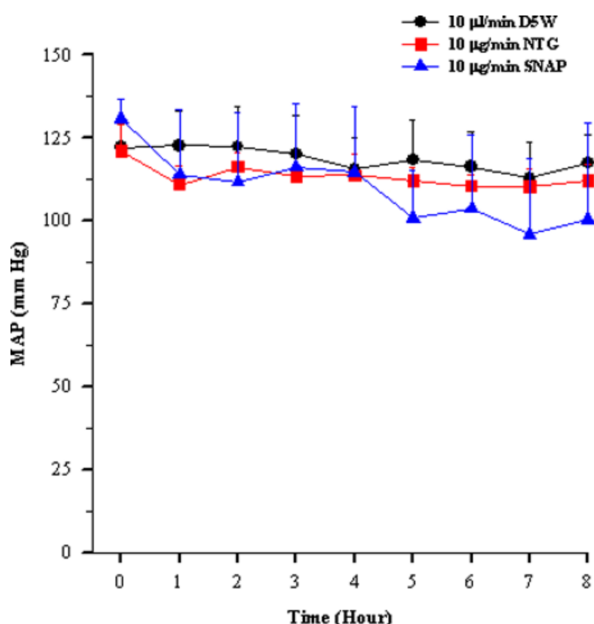

Figure 1. The effects of 10 µg/min NTG or SNAP continuous infusion on MAP. (●): 10 µL/min D5W control infusion, (■): 10 µg/min NTG infusion, (▲): 10 µg/min SNAP infusion. Data are expressed as mean ± SD, n = 4-6.

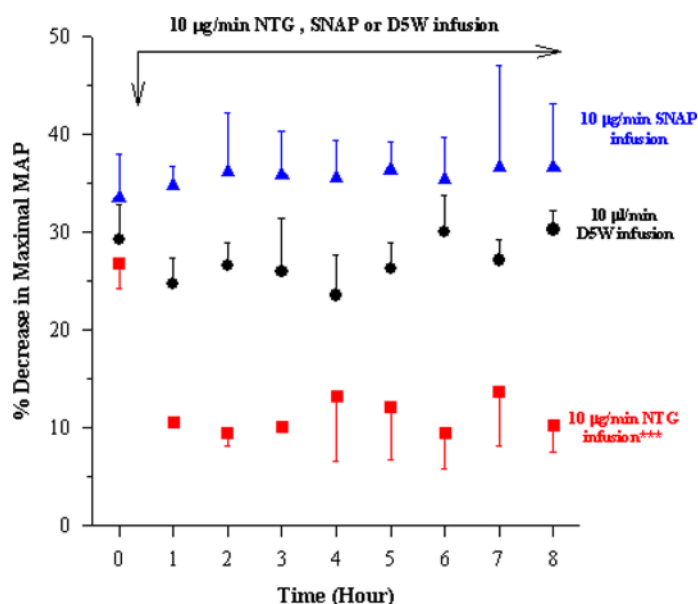

Figure 2. The effects of NTG or SNAP infusion on the peak MAP response of the hourly 30 µg NTG IV bolus challenge dose. (●): 10 µL/min D5W control infusion, (■): 10 µg/min NTG infusion, (▲): 10 µg/min SNAP infusion. Data are expressed as mean ± SD, n = 4-6. *** P < .001 vs corresponding 0 hr response, ANOVA.

period. In the NTG-infused group, attenuation in the hypotensive effect of the NTG CD was observed from the first hour of NTG infusion (*P* < .001, ANOVA), confirming the development of nitrate vascular tolerance. In the presence of SNAP infusion, repeated NTG bolus CD produced a consistent decrease in peak MAP of about 33%, and none of the values obtained during infusion was different from its corresponding baseline response at zero hour (*P* > .05, ANOVA). These results indicated that SNAP infusion did not diminish the MAP response of the NTG CD, suggesting the absence of cross-tolerance between NTG and SNAP in normal conscious rats.

Figure 3 shows the maximal MAP response of the 30-µg SNAP bolus CD in the presence of SNAP infusion (10 µg/min). Similar to the MAP response produced by the NTG CD, the 30-µg SNAP bolus dose produced a decrease of 30.4% ± 6.5% in maximal MAP prior to SNAP infusion (*P* > .05 vs NTG, Student *t*-test). At 2, 4, 6, and 8 hours after the start of SNAP infusion, the SNAP CD still produced a similar MAP response as that observed at zero hour (*P* > .05, ANOVA). These results suggested that SNAP did not produce self-tolerance in MAP response in our animal model.

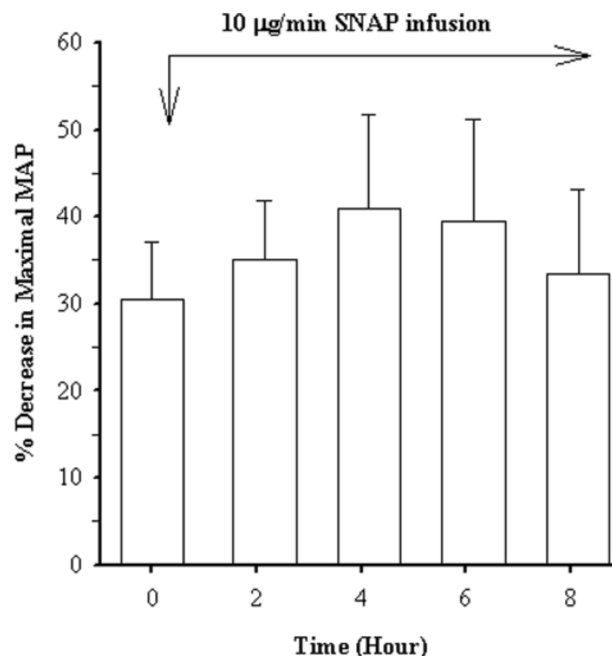

Figure 3. The effects of 10 µg/min SNAP continuous infusion on the peak MAP response of the 30-µg SNAP IV bolus challenge dose. Data are expressed as mean ± SD, n= 4-6.

Differential Vascular Gene Expression Patterns Induced by NTG and SNAP Infusions

In preliminary studies using the same RNA sample, we found that stripping and membrane crossover had no apparent effects on gene expression so long as the microarray membranes were not stripped more than 4-5 times. Table 2 lists the general descriptive statistics for the 2 sets of microarrays (NTG vs D5W or SNAP vs D5W) used in our study. Similar average background intensities were observed between NTG vs D5W and SNAP vs D5W. The normalized average intensity for the 2 sets of microarrays was also similar, with signals ranging from 1827 to 1979 arbitrary units, indicating that NTG or SNAP treatment did not cause a global up- or down-regulation of the genes spotted on the microarray filter. In D5W control animals, a wide range of intensities was observed for the 5531 genes, indicating that these genes were differentially expressed in the rat aorta in the absence of drug treatment. The mean coefficient of variation (% CV) was found to be fairly similar between treated and control membranes. The microarray data obtained for the genes were quite variable, as indicated by the wide range of CVs, ranging from 1% to 200% for the 5531 genes. However, at most only 0.5% of the gene signals had % CV greater than 100 for both sets of microarray filters. The degrees of variability that we observed were consistent with other reported studies employing the gene microarray technique.[29]

Application of *t*-statistics to the microarray data revealed that the expression of 447 genes was significantly altered by NTG treatment vs control, of which 252 were up-regulated and 195 were down-regulated. In comparison, SNAP infusion led to alteration in the expression of 67 genes, of which 14 were up-regulated and 53 were down-regulated. Application of the more stringent permutation-adjusted *t*-statistic to the NTG data showed that a subset of 290 genes exhibited the highest rank of the *t*-statistic among all 35 possible permutated data sets. Of these, 131 genes were significantly higher, and 159 genes were lower, after NTG treatment when compared to D5W control. In comparison, application of the permutation-adjusted *t*-statistic to the SNAP infusion data produced 41 significantly altered genes, of which 7 were up-regulated and 34 were down-regulated.

The "called" genes after using the permutation-adjusted statistical method were further examined. Table 3 and 4 give listings of the specific genes that were up-regulated or down-regulated, respectively, as a result of NTG infusion. Of the known vascular genes that had been significantly up-regulated, the changes ranged from 130% to 226% (Table 3). In comparison, the significantly down-regulated genes generally showed about a 2-fold decrease in expression. After SNAP treatment, 5 of 7 up-regulated genes were ESTs (expressed sequence tags), while the remaining 2 genes encode the basement membrane-associated chondrotin proteoglycan Bamacan (SNAP/D5W = 1.45) and mannose 6-phosphate/insulin-like growth factor II receptor (MAP/IGF2r, SNAP/D5W = 1.80). The known genes that were down-regulated by SNAP treatment are listed in Table 5. Interestingly, from our analysis, there were no common genes that were altered by both NTG and SNAP treatment.

DISCUSSION

Hemodynamic Differences Between NTG and SNAP

The present study showed that NTG and SNAP exerted differential hemodynamic tolerance properties as well as gene expression patterns after in vivo treatment. This observation is consistent with the view that the pharmacological actions of NO donors are not identical, even though they all release NO as the obligatory intermediate. The presence of MAP tolerance was clearly demonstrated for NTG, while SNAP showed no apparent tolerance development.

The absence of in vivo cross-tolerance between NTG and SNAP, and self-tolerance toward SNAP, are clearly demonstrated in Figure 2 and 3. These results are consistent with those of Bauer et al,[17] who showed in a rat model of congestive heart failure that NTG hemodynamic tolerance, measured as % change in left ventricular end-diastolic pressure, was observed within 5 hours of continuous NTG infusion while SNAP produced little apparent tolerance. In addition, our in vivo studies are consistent with previous in vitro findings showing that SNAP produced no apparent tolerance as measured by vascular relaxation[16] and cGMP production.[30]

The apparent differences in the hemodynamic properties between NTG and SNAP may in part arise from the differences in NO liberation from these 2 NO donors. NTG requires metabolic activation and cofactors such as thiols to release NO while S-nitrosothiol metabolism to transfer NO may require enzymes such γ-glutamyl transpeptidase[15] or glutathione-dependent formaldehyde dehydrogenase.[31] Since NTG is highly lipophilic, it is generally assumed that NO release from NTG occurs intracellularly, which then acts on vascular smooth muscles. During tolerance development there might be a down-regulation of the enzymes that are involved in NTG metabolism such as cytochrome P-450 and GST. In contrast, SNAP is a much more polar compound than NTG,[32] and it is generally assumed that this agent can release NO in the extracellular space, and undergoes a transnitrosation process by which NO is transferred from one molecule to the next via cysteine residues in pro

Table 2. General Descriptive Statistics of the 2 Sets of Microarray Filters, n = 4 Replicates for Each Set. Key: a.u.= arbitrary unit.

	D5W vs NTG		**D5W vs SNAP**	
	D5W	NTG	D5W	SNAP
Mean background intensity (a.u.)	11.4	10.9	27.6	18.7
Mean normalized intensity (a.u.)	1979	1827	1978	1955
Normalized intensity range (a.u.)	465 - 119929	828 - 42372	225 - 27185	328 - 112272
Mean CV (%)	28.5	39.3	53.8	40.5
range of % CV	1.4 - 184	3.3 - 156.2	1.4-113.2	2.3 - 140.1
% genes that exhibited CV > 100%	0.23	0.33	0.52	0.21

teins.[30] Recently, Tseng et al[4] reported that NTG and SNAP exhibited a differential sensitivity toward inhibition by 1H-[1,2,4] oxadiazolo [4,3-α]quinoxalin-1-one (ODQ, an inhibitor of soluble guanylyl cyclase, sGC). These authors suggested that in addition to the activation of the heme-site on sGC, SNAP can activate the sulfhydryl-site on sGC, leading to vasodilation.[4] The differences between NTG and SNAP in metabolic activation and sGC activation may contribute to the differential hemodynamic effects observed in our present study.

Statistical Issues in the Analysis of Gene Array Data

A simple technique that has been applied for comparisons of microarray data involved the identification of genes with a 2-fold or higher difference between the mean intensity for the group. However, this approach fails to account for sample variation and possibly leads to the false positives when a data set has considerable variability. For example, Miller et al[33] have shown, via a simulation study using 10 000 genes, that the ratios of 450 genes can be higher than 2 by chance alone with a 35% of CV. In addition, this ratio-based approach ignores the fact that a difference less than 2-fold can also elicit meaningful biological effects. As an alternative approach, the parametric *t*-statistic has been used in the data analysis of DNA microarrays. This method, however, assumes normality and constant variance, which may not be always appropriate for gene expression data in microarrays. We therefore calculated a permutation-adjusted *t*-statistic in order to account for the unequal variance between genes that showed low vs high expression levels. By comparing the *t*-statistic of a given data set against the *t*-statistic distribution of all possible permutated data sets, we further assessed the likelihood of obtaining a given significant *t*-statistic observed by chance alone.

Although the permutation-adjusted *t*-statistic identified 290 genes that are significant, these genes may still include some false positives. Given the extreme number of multiple comparisons, the use of probability values to assign significance in microarray studies leads to the high occurrence rate of false positives (the family-wise Type I Error, FWE). A number of statistical approaches are available to control this false-positive rate resulting from multiple comparisons. For example, the Bonferroni correction is a single step method to adjust the significance criteria in multiple hypothesis testing (the adjusted *P* value = .00001 for multiple testing of 5531 genes). However, this correction is often found to be overly conservative for microarray data analysis and has very low power when the number of tests is high. Indeed, this correction method identified no genes to be significantly different after NTG treatment. In addition, the Bonferroni correction, like other multiple-comparison corrections for single-inference procedures, assumes that each test is independent of the other. This is unlikely to be the case in gene expression studies from biological samples, since various mechanistic pathways interact with many others.

Using a statistical resampling approach, Westfall and Young's step-down adjustment method has been adapted for the analysis of DNA microarray.[34] This step-down correction method indicated that a subset of 55 genes was significantly different. Of these, 24 were significantly higher and 31 were significantly lower in NTG treatment than D5W treatment. Recently, Tusher et al[28] have published another method for microarray data analysis, called Significance Analysis of Microarrays (SAM), which accounts for multiple comparisons during the analysis of microarray data sets. This method has an advantage of estimating the percentage of wrongly significant genes, the false discovery rate (FDR), by using permutations of the repeated measurements. The authors have reported that the step-down adjustment method of Westfall and Young[34] was still too stringent for

Table 3. Genes That Were Significantly Up-regulated by NTG Treatment vs Control, As Shown by the Adjusted *t*-statistic*

Accession #	Gene Title	Ratio NTG/D5W
AA818896	cytochrome P-450j mRNA	1.53
AI138145	cyclic GMP stimulated phosphodiesterase (PDE2A2)	1.83
AA964809	SNF1-related kinase	1.77
AI045179	STAT3 protein	2.17
AI072547	tyrosine phosphatase CBPTP	2.26
AI070051	Transcription factor IIIC alpha-subunit	1.91
AA956939	tyrosine kinase p72 (syk)	1.81
AA955510	monoamine oxidase B (Maobf3)	1.30
AI144995	Electrogenic Na+ bicarbonate cotransporter (NBC)	2.04
AI138126	Amylin	1.60
AI144744	prostaglandin D synthetase	1.55
AA925908	phospholipase A2 precursor	1.74
AA955059	pancreatic phospholipase A-2	1.53
AI071141	insulin receptor substrate-3 (IRS-3)	1.73
AA899448	ribosomal protein S3a	1.72
AA817697	ADP-ribosylation factor-like protein ARL184	1.72
AI060106	Hepsin	1.52
AA997521	heparin-binding fibroblast growth factor receptor 2 (extracellular domain)	1.91
AA900301	adult brain APEX nuclease	1.80
AA866248	AIR carboxylase-SAICAR synthetase	1.89
AA874911	assembly protein (AP50) associated with clathrin-coated vesicles	1.61
AI137736	calmodulin (RCM3)	1.57
AI137921	CD3d mRNA for T3 delta protein	1.54
AA926170	mitochondrial acetoacetyl-CoA thiolase	1.56
AI137246	140-kD NCAM polypeptide	1.84
AI030627	pancreatitis associated protein (pap)	1.64
AI145494	synapsin 2a	1.81
AI059479	Tamm-Horsfall protein	1.71
AI071947	cortactin-binding protein 1	2.00
AI044110	lamina associated polypeptide 2 (LAP2)	1.93
AI145215	ETR-R3b protein, alternatively spliced isoforms	1.50
AA964507	ID2 protein	1.72
AI071529	p27	2.10
AI144709	RGS8	1.67
AA900189	mRNA similar to cdc37	1.85
AA956438	Mxi1 protein (rMxi1)	2.18
AI071000	Nclone10	1.46
AA858569	neuronatin alpha mRNA	1.43
AI030613	RJTB	1.71
AA964945	RNA polymerase I 127 kDa subunit	1.76
AA998713	urokinase receptor	1.69
AA956238	Vitronectin	2.14

*This list did not include genes encoding 85 ESTs.

Table 4. Genes That Are Significantly Down-regulated by NTG Treatment vs Control, As Shown by the Adjusted *t*-statistic*

Accession #	Gene Title	Ratio NTG/D5W
AA818412	cytochrome p-450, phenobarbital-inducible,	0.50
AA818339	liver glutathione S-transferase Ya subunit, clones pGTR112 and pGTB38	0.52
AA924224	aldosterone synthase cytochrome P-450	0.62
AA957669	Dual Specificity Yak1-related Kinase (Dyrk)	0.53
AA866273	protein phosphatase 2A-beta catalytic subunit	0.53
AA858945	Ras-related protein (rheb)	0.56
AA924050	G protein-coupled receptor kinase GRK6a	0.57
AA925506	G protein gamma subunit (gamma7 subunit)	0.55
AI030295	GTP cyclohydrolase I feedback regulatory protein	0.47
AA817965	CRP2 (cysteine-rich protein 2)	0.53
AA818791	cretine kinase-B (CKB)	0.61
AA996409	urate oxidase 2	0.53
AA925006	adult brain NADH:ubiquinone oxidoreductase	0.54
AA860001	flavin-containing monooxygenase 1 (FMO-1)	0.55
AA924524	12-lipoxygenase	0.58
AA955106	aldehyde dehydrogenase (ALDH)	0.58
AA955455	Ceruloplasmin	0.57
AA924618	high molecular weight (HMW) K-kininogen	0.47
AA925580	stannin	0.48
AA858694	TTF-1 for thyroid nuclear factor 1	0.49
AA818082	myr5	0.49
AA964044	apolipoprotein A-I (apoA-I)	0.50
AA817928	thy-1 antigen	0.50
AA901070	contrapsin-like protease inhibitor related protein (CPi-26)	0.50
AA859471	7-dehydrocholesterol reductase	0.50
AA924611	CD30	0.51
AA818799	CD25	0.53
AA925584	lymphocyte antigen CD5	0.53
AA956083	F1-ATPase alpha subunit (EC 3.6.1.34)	0.53
AA819385	Ryudocan	0.55
AA859846	cytoplasmic beta-actin	0.55
AI070076	preprocathepsin D (EC 3.4.23.5)	0.55
AA819571	leukocyte antigen MRC-OX44	0.55
AA819022	beta defensin-2	0.55
AI145198	cytoplasmic dynein 74 kD intermediate chain	0.55
AA924695	receptor binding factor-1	0.55
AA874841	Androgen binding protein (ABP)	0.56
AA858662	14-3-3 zeta isoforms	0.56
AA817804	PCB-binding protein	0.58
AA925943	branched chain alpha-keto acid dehydrogenase E1-subunit (BCKDHB)	0.59
AA899852	epithelin 1 and 2	0.60
AA924912	Ribosomal protein L6	0.61
AI029343	preoptic regulatory factor-1 (PORF-1)	0.62
-	cyclic nucleotide-gated cation channel	0.63
AA818413	TRPM-2	0.63
AA925597	calpain II 80 kDa subunit	0.65

*This list did not include genes encoding 113 ESTs.

their data, while SAM allowed them to identify a subset of genes with an acceptable FDR.[28] Using a microarray analysis package provided by these authors (downloaded from http://www-stat.stanford.edu/~tibs/SAM/index.html), we showed that a subset of 231 genes (all down-regulated) was significant at an estimated FDR of <1%. This method appeared to be less stringent than the step-down correction method and also allowed us to adjust the FDR, which in turn affected the number of genes that could be called significantly different. However, the estimated values for FDR appeared to be distributed rather unevenly and more than 2000 genes were identified as significantly different at ~7.5% FDR. At <1% FDR, 159 out of 231 genes were found to be overlapping with the genes that were identified as significantly down-regulated by the permutation-adjusted *t*-statistic that we employed. Therefore, SAM, even at <1% FDR, identified more genes to be significantly down-regulated than the permutation-adjusted *t*-statistic.

Table 5. Genes That Are Significantly Down-regulated by SNAP Treatment vs Control, As Shown by the Adjusted *t*-statistic

Accession	Gene Title	Ratio (SNAP/D5W)
AI045079	R.norvegicus mRNA for acidic ribosomal protein P0	0.760
AI043748	R.norvegicus mRNA for Fc gamma receptor	0.417
AA965022	R.norvegicus mRNA for macrophage metalloelastase (MME)	0.541
AA925907	R.norvegicus mRNA for muscle LIM protein	0.480
AA900928	Rat aspartate aminotransferase	0.550
AA924761	Rat Fc-gamma receptor	0.387
AI045139	Rat mRNA for inositol 1,4,5-triphosphate 3-kinase	0.414
AA817825	Rat peptidyl-glycine alpha-amidating monooxygenase (rPAM-1)	0.434
AA996424	Rat tropoelastin mRNA, 3' end	0.293
AI058642	Rattus norvegicus growth hormone-releasing hormone (GHRH)	0.570
AA999001	Rattus norvegicus hematopoietic cell tyrosine kinase (hck)	0.509
AA858801	Rattus norvegicus nuclear factor kappa B p105 subunit mRNA, 3' end	0.431
AI144658	Rattus norvegicus paired-like homeodomain transcription factor (DRG11)	0.690
AA817946	Rattus norvegicus rsec15 mRNA	0.470
AA859083	Rattus norvegicus Sprague Dawley protein kinase C receptor	0.518
AI045067	Rattus norvegicus water channel protein (AQP3) gene	0.578

These results suggest that the approach using the permutation-adjusted *t*-statistic can identify genes that are more likely to be differentially regulated without substantially increasing the false discovery rate, either false positives or negatives, compared to the other methods. Therefore, we chose the permutation-adjusted *t*-statistic for our data analysis. The field of bioinformatics relating to interpretation of gene microarray data is at present in its nascent stage. Future development in this field, accompanied by acquisition of more experimental data, will lead to a more concrete paradigm for analyzing these data.

Changes in Vascular Gene Expression and Mechanisms of Nitrate Tolerance

The transcriptional changes shown for a number of genes (Table 3 and 4) appeared to be consistent with literature reports documenting the presence of specific regulatory changes associated with nitrate tolerance. For example, there was an increased expression of genes for cGMP-stimulated phosphodiesterase (NTG/D5W = 1.83),[8] an enzyme responsible for the breakdown of cGMP, while the decrease in gene expression was observed with metabolic enzymes such as CYP450 (phenobarbital-inducible, NTG/D5W = 0.50) and glutathione S-transferase (Ya subunit, NTG/D5W = 0.52).[35,36]

In addition, a number of genes that are involved in cellular signaling were altered by NTG treatment. For example, the expression levels of genes encoding various kinases and phosphatases were found to be altered: tyrosine phosphatase (CBPTP, NTG/D5W = 2.26), protein phosphatase (2A-beta subunit, NTG/D5W = 0.53), and G protein-coupled receptor kinase (GRK6a, NTG/D5W = 0.57). Differentially expressed genes also included transcription factor (IIIC alpha-subunit, NTG/D5W = 1.91), STAT3 protein (NTG/D5W = 2.17), and cysteine-rich protein 2 (CRP2, NTG/D5W = 0.53). These findings support the hypothesis that the pharmacological effects of NTG are mediated by alterations in signaling events that follow transcriptional changes of many related genes. Some of these genes appeared to have some relevance to the mechanisms of NTG action. For example, signaling pathways involving the STAT family of transcription factors have been shown to contribute to the cardioprotective effect during myocardial ischemia,[37] for which NTG is widely used. CRP2 has been recently identified as a novel substrate for cGMP kinase I,[38] which is a major target of cGMP in smooth muscle.

Importantly, the differentially expressed genes included many genes associated with cellular oxidation/reduction, for example, genes coding various oxidases and reductases (Table 3 and 4). In recent years, oxidation by reactive oxygen and nitrogen species has been increasingly recognized as an important signaling and regulatory mechanism.[39,40] Our observations are consistent with the view that vascular nitrate tolerance might be associated with oxidative stress[9] and possibly oxidative protein modification.

Although the GeneFilters® membranes (GF300) were not customized to monitor the expression of genes for vascular signaling, our results appeared to provide some interesting leads that can be used to probe the possible mechanisms of NTG actions. For example, ceruloplas-

min (NTG/D5W = 0.57) belongs to the family of multicopper oxidases and has been suggested as an important risk factor predicting myocardial infarction and cardiovascular diseases.[41,42] GTP cyclohydrolase I feed-back-related protein (NTG/D5W = 0.47) is the rate-controlling enzyme in the production of tetrahydrobiopterin, an essential cofactor for NO synthesis.[43]

Consistent with the results found in the in vivo hemodynamic study, the cDNA microarray study also revealed differential gene regulations by NTG and SNAP. Vascular nitrate tolerance appeared to be accompanied by alterations in the expression of many genes. SNAP produced no apparent hemodynamic tolerance and affected the change of a smaller number of vascular genes. In contrast to the results observed with NTG, only 2 "known" genes were induced by SNAP. Thus, it can be argued that these changes were unlikely to be derived from the NO action of SNAP, since NTG (which also produced NO) did not elicit these changes.

Mechanistic interpretations can also be attached to those known genes that were down-regulated by NTG. Intracellular thiol depletion has long been suggested as a mechanism of nitrate tolerance, since thiols are believed to be important in NTG metabolism.[6] This mechanism is consistent with our observation that the gene encoding for cysteine-rich protein was repressed by NTG. In addition, metabolic inactivation of GST and cytochrome P-450, 2 known NTG metabolizing enzymes, had also been suggested as a mechanism of nitrate tolerance.[36] Indeed we found that the genes encoding for these enzymes were down-regulated by NTG treatment but not by SNAP.

It is recognized that caution should be exercised when interpreting data from microarrays since these arrays primarily serve as a first line screening method for drug-induced effects. Results obtained from these studies should be further confirmed either by traditional methods such as Northern and Southern blot analyses or by quantitative real time PCR. Nevertheless, using this new technique, we have demonstrated for the first time an apparently extensive effect of vascular nitrate tolerance on gene expression. Consistent with the differential hemodynamic effects of NTG and SNAP, we also observed differential gene expression patterns induced by these 2 NO donors, suggesting that altered gene expression in the vasculature may play a role in nitrate tolerance.

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