Effects of ADMA upon Gene Expression: An Insight into the Pathophysiological Significance of Raised Plasma ADMA

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Competing Interests: University College London holds patents on DDAH as a drug target (not relevant to the present study).

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Abbreviations: ADMA, asymmetric dimethylarginine; BMP, bone morphogenetic protein; BMPRTA, bone morphogenetic protein receptor 1A; BMP2K, bone morphogenetic protein 2 inducible kinase; DDAH, dimethylarginine dimethylaminohydrolase; HCAEC, human coronary artery endothelial cells; L-NIO, L-5-(1-iminoethyl)ornithine; mβ-actin, murine β-actin; mBMP2K, murine bone morphogenetic protein 2 inducible kinase; mPRMT3, murine protein arginine methyltransferase 3; NO, nitric oxide; NOS, nitric oxide synthase; PRMT, protein arginine methyltransferase; Q-PCR, quantitative PCR; RgL27, ribosomal protein L27; RgS11, ribosomal protein S11; SCAMP1, secretory carrier membrane protein 1; SDMA, symmetric dimethylarginine; Smad5, SMA-related protein 5

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ABSTRACT

Background

Asymmetric dimethylarginine (ADMA) is a naturally occurring inhibitor of nitric oxide synthesis that accumulates in a wide range of diseases associated with endothelial dysfunction and enhanced atherosclerosis. Clinical studies implicate plasma ADMA as a major novel cardiovascular risk factor, but the mechanisms by which low concentrations of ADMA produce adverse effects on the cardiovascular system are unclear.

Methods and Findings

We treated human coronary artery endothelial cells with pathophysiological concentrations of ADMA and assessed the effects on gene expression using U133A GeneChips (Affymetrix). Changes in several genes, including bone morphogenetic protein 2 inducible kinase (BMP2K), SMA-related protein 5 (Smad5), bone morphogenetic protein receptor 1A, and protein arginine methyltransferase 3 (PRMT3; also known as HRMT1L3), were confirmed by Northern blotting, quantitative PCR, and in some instances Western blotting analysis to detect changes in protein expression. To determine whether these changes also occurred in vivo, tissue from gene deletion mice with raised ADMA levels was examined. More than 50 genes were significantly altered in endothelial cells after treatment with pathophysiological concentrations of ADMA (2 μM). We detected specific patterns of changes that identify pathways involved in processes relevant to cardiovascular risk and pulmonary hypertension. Changes in BMP2K and PRMT3 were confirmed at mRNA and protein levels, in vitro and in vivo.

Conclusion

Pathophysiological concentrations of ADMA are sufficient to elicit significant changes in coronary artery endothelial cell gene expression. Changes in bone morphogenetic protein signalling, and in enzymes involved in arginine methylation, may be particularly relevant to understanding the pathophysiological significance of raised ADMA levels. This study identifies the mechanisms by which increased ADMA may contribute to common cardiovascular diseases and thereby indicates possible targets for therapies.
Introduction

Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of all nitric oxide synthase (NOS) isoforms [1]. It is synthesised by the action of protein arginine methyltransferases (PRMTs), and following proteolysis, free ADMA is released into the cell cytosol and thence into plasma. Circulating concentrations of ADMA are increased in patients with renal failure [1], pulmonary hypertension, heart failure, hypercholesterolemia or a wide range of other cardiovascular risk factors [2–6]. In patients with end-stage renal failure, the plasma levels of ADMA predict mortality and cardiovascular outcome [7], and in a cohort of otherwise healthy Finnish men, those with the highest levels of ADMA had an increased risk of acute coronary events [8]. Increased circulating ADMA in pregnant women predicts an increased risk of pre-eclampsia and intrauterine growth retardation [9].

Despite these clinical observations and the increasing excitement surrounding the use of ADMA as a risk marker for vascular disease [3,7], it is still not clear whether ADMA has a causal role in pathophysiology. It has been argued that the concentration of ADMA in plasma is too low to be an effective inhibitor of NOS, and that the usual concentrations of arginine in cells should overcome any inhibitory effects of ADMA on NOS [10]. In order to determine how ADMA might exert effects on endothelial cells and produce pathology, we assessed the effects of ADMA on gene expression in human coronary endothelial cells.

Methods

Cell Culture

Human coronary artery endothelial cells (HCAEC) were purchased from Promocell and grown according to the manufacturer’s instructions. HCAEC in 75-cm² flasks at 70% confluence (passage 3 or 4) were treated for 24 h with complete media supplemented with asymmetric dimethylarginine (N⁵,N⁷-dimethyl-L-arginine; ADMA; 0, 2, or 100 μM;...
Merck Biosciences, United Kingdom). This was repeated on three separate occasions with different batches of cells. RNA from each study was used as described below for GeneChip (Affymetrix, Santa Clara, California, United States) analysis. Our strategy for the GeneChip and subsequent analysis is outlined in Figure 1.

**GeneChip Experiments**

ADMA-treated HCAEC from T75 flasks were harvested in 7.5 ml of TRIzol (Invitrogen, Carlsbad, California, United States), and total RNA was extracted; cDNA and subsequent cRNA synthesis were prepared as previously described [11]. The quality of the biotin-labelled cRNA transcripts was determined using a Bioanalyser 2100 (Agilent Technologies, Palo Alto, California, United States). Purified cRNA (15 μg) was fragmented and hybridised to human U133A GeneChips according to Affymetrix standard protocols (http://www.affymetrix.com). Labelled GeneChips were scanned, using a confocal argon ion laser (Agilent Technologies).

**GeneChip Data Analysis**

The U133A GeneChip contains oligonucleotides derived from approximately 22,000 human transcripts and includes control bacterial genes from approximately 22,000 human transcripts and includes control bacterial genes is gene-specific. Standard curves were constructed for each gene using the same number of ADMA-treated HCAEC from T75 flasks were harvested in 7.5 ml of TRIzol (Invitrogen, Carlsbad, California, United States), and total RNA was extracted; cDNA and subsequent cRNA synthesis were prepared as previously described [11]. The quality of the biotin-labelled cRNA transcripts was determined using a Bioanalyser 2100 (Agilent Technologies, Palo Alto, California, United States). Purified cRNA (15 μg) was fragmented and hybridised to human U133A GeneChips according to Affymetrix standard protocols (http://www.affymetrix.com). Labelled GeneChips were scanned, using a confocal argon ion laser (Agilent Technologies).

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and murine protein dimethylaminohydrolase (Amersham Biosciences). The 0.05, 56 genes were identified as having results are (mPRMT3)

RpS11

bone morphogenetic protein receptor 1A

were purified and oligonucleotides (Table 2) using T4 polynucleotide kinase.

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mPRMT3, detection and

6.

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individual arrays are shown for each treatment compared with untreated cells with 11 genes changing with both concentrations of ADMA. Individual arrays are shown for each treatment group with blue representing low expression, and red high expression in response to 2

labelling kit (Roche).

labelled with Redivue deoxycytidine 5

arginine methyltransferase 3

protein 2 inducible kinase

Western Blotting

HCAEC were treated with either 0, 2, or 100 µM ADMA for 24 h in 6-well plates and harvested in lysis buffer as described previously [11], the protein concentrations of the lysates were determined by protein assay (Bio-Rad, Hercules, California, United States), and cell lysates were resolved by 12% SDS polyacrylamide gel electrophoresis with equal amounts of protein loaded into each lane. Anti-PRMT3 (Upstate Biotechnology, Charlotte, Virginia, United States) and Anti-BMP2K (Orbigen, San Diego, California, United States) were used with anti-rabbit secondary antibody coupled to horse-radish peroxidase and detected with the ECL+ detection system (Amersham Pharmacia, Piscataway, New Jersey, United States). Densitometry of the bands was determined, and results are shown as the mean densitometry, where n = 4 with inset of a typical Western blot.

Pathway Mapping and Gene Ontology Analysis

In order to determine whether ADMA had affected expression of genes in pathways related to the genes identified on the initial analysis, lists of genes that were changed more than 1.7-fold compared to control (irrespective of p-value) were examined using Gene Ontology (Affymetrix) data mining for biological process (at level 3), and Expression Analysis Systematic Explorer (EASE) biological theme analysis were conducted online at http://david. niaid.nih.gov using DAVID [14]. DAVID-EASE [15] generates an EASE score predicting the likelihood of genes mapping to specific biological processes (determined by Gene Ontology consortium) from a given list of changed genes, therefore enabling global themes in gene expression following ADMA treatment to be identified [15].

Dimethylarginine Dimethylaminohydrolase 1 Gene Deletion Mice

ADMA is metabolised to citrulline and dimethylamine by the action of dimethylarginine dimethylaminohydrolase (DDAH). We have created knockout mice that lack DDAH1. DDAH1 heterozygous knockout mice (details to be published elsewhere) have approximately 2-fold higher plasma ADMA levels compared to wild-type litter-mates and thus provide an excellent model to test the effects of moderately raised ADMA levels in vivo. Northern blotting was carried out using RNA extracted from the brain, heart, and kidney of 12–14-wk-old DDAH1 heterozygous knockout and wild-type litter-mates with probes for mBMP2K and mPRMT3, and results are expressed relative to m-actin.

Statistical Analyses

Q-PCR, Northern blot, and Western blot densitometry data for the treated HCAEC was analysed by one-way analysis of variance (ANOVA) coupled to Bonferroni posttest, and the Bonferroni posttest p-values are reported. The Northern blots from the DDAH1 gene deletion mice were compared with unpaired t-test and the p-values are reported.

Results

Changes in HCAEC Gene Expression in Response to ADMA on U133A GeneChips

A total of 979 genes changed in expression between ADMA-treated and control cells. Following the Welch t-test with a cutoff of p < 0.05, 56 genes were identified as having shown a statistically significant change between the untreated and 2-µM ADMA-treated cells, and 86 genes changed between
Effects of ADMA upon HCAEC Viability

Basal levels of ADMA and SDMA in HCAEC media were $0.17 \pm 0.01$ μM and $0.22 \pm 0.02$ μM, respectively, and arginine levels exceeded 300 μM. No changes were observed in the untreated and 100-μM ADMA greater than 1.7-fold; 11 genes showed statistically significant changes at both concentrations of ADMA compared to untreated cells (Figure 2A; Tables 3 and 4).

Table 3. Genes That Changed by More Than 1.7-Fold in 2 μM ADMA Treated HCAEC Compared to Untreated

| Affymetrix ID | Gene Title | Gene Symbol | Fold Change | p-Value |
|---------------|------------|-------------|-------------|---------|
| 213350_at     | Ribosomal protein S11 | RpS11      | 8.065       | 0.0334  |
| 222364_at     | EST        | RpL27       | 5.780       | 0.0168  |
| 210642_at     | Ribosomal protein L27 |           | 4.386       | 0.0481  |
| 216859_x_at   | Golgi-specific brefeldin A resistance factor 1 | GEF1      | 3.968       | 0.00706 |
| 212952_at     | Calreticulin | CALR       | 2.959       | 0.0472  |
| 204569_at     | Intestinal cell (MAK-like) kinase | ICK      | 2.778       | 0.0491  |
| 203294_s_at   | Lectin, mannose-binding, 1 | LMAN1     | 2.740       | 0.0433  |
| 210407_at     | Protein phosphatase 1A (formerly 2C), Magnesium-dependent, α-isom | PPMA1     | 2.674       | 0.036   |
| 205636_at     | SH3GL3 protein | SH3GL3     | 2.404       | 0.00324 |
| 38290_at      | Regulator of G-protein signalling 14 | RG514     | 2.347       | 0.0235  |
| 208995_s_at   | Peptidyl-prolyl isomerase G (cytochrome G) | PPIG      | 2.315       | 0.0146  |
| 214716_at     | BMP2 inducible kinase | BMP2K     | 2.304       | 0.00128 |
| 213610_s_at   | Hypothetical protein MGC2610 | MGC2610  | 2.299       | 0.0435  |
| 212209_at     | Thyroid hormone receptor associated protein 2 | THRAP2    | 2.137       | 0.00562 |
| 214697_s_at   | ROD1 regulator of differentiation 1 (Schizosaccharomyces pombe) | ROD1      | 2.132       | 0.02    |
| 213732_at     | Transcription factor 3 | TCF3      | 2.119       | 0.0462  |
| 220112_at     | Hypothetical protein FLJ11795 | FLJ11795 | 2.114       | 0.0623  |
| 219785_s_at   | F-box protein 31 | FBXO31     | 2.079       | 0.00429 |
| 206928_at     | Zinc finger protein 124 (HIF-16) | ZNF124    | 2.045       | 0.0274  |
| 221903_s_at   | Cylindromatosis (turban tumor syndrome) | CYLD      | 2.024       | 0.0221  |
| 204662_at     | CP110 protein | CP110      | 2.012       | 0.0165  |
| 217246_at     | KARP-1-binding protein | KAR1     | 1.980       | 0.0379  |
| 217540_at     | Transcribed locus, moderately similar to NP_055301.1 neuronal thread protein | —        | 1.946       | 0.00294 |
| 202364_at     | MAX interacting factor | MIO1      | 1.919       | 0.0268  |
| 204285_s_at   | Phorbol-12-myristate-13-acetate-induced protein 1 | PMAP1     | 1.916       | 0.0443  |
| 204010_s_at   | v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog | KRAS      | 1.905       | 0.046   |
| 203205_s_at   | Fasciculation and elongation protein zeta 2 (zygin II) | FEZ2      | 1.862       | 0.028   |
| 201242_s_at   | ATPase, Na+/K+ transporting, β-1 polypeptide | ATP1B1    | 1.845       | 0.0391  |
| 205416_s_at   | Taxasin 3 | ATXN3      | 1.845       | 0.00666 |
| 218096_at     | 1-Acetylglcero-3-phosphate O-acetyltransferase 5 (lysophosphatidic acid acyltransferase, c) | AGPAT5    | 1.838       | 0.00518 |
| 218930_s_at   | Hypothetical protein FLJ11273 | FLJ11273 | 1.828       | 0.0495  |
| 214700_s_at   | RAP1 interacting factor homolog (yeast) | RIF1      | 1.818       | 0.00974 |
| 215203_at     | Golgi antiotigen, golgin subfamily a, 4 | GOLGA4    | 1.812       | 0.0128  |
| 202225_at     | V-erb B2 avian erythroblastosis virus CT10 oncogene homolog (avian) | CRK       | 1.805       | 0.0429  |
| 209318_s_at   | Pleiomorphic adenoma gene-like 1 | PLAG1     | 1.805       | 0.0433  |
| 202906_s_at   | Nijmegen breakage syndrome 1 (nibrin) | NBS1      | 1.773       | 0.0492  |
| 215948_s_at   | Zinc finger protein 237 | ZNF237    | 1.761       | 0.042   |
| 203202_at     | HIV-1 rev binding protein 2 | HVRB2     | 1.718       | 0.0449  |
| 215412_s_at   | Postmetabolic segregation increased 2-like | PMSCL2    | 1.706       | 0.0483  |
| 219021_s_at   | Ring finger protein 121 | RFC15D1   | 1.588       | 0.0311  |
| 208704_at     | Amyloid β (Aβ) precursor-like protein 2 | APP2L2    | 0.582       | 0.0433  |
| 211590_s_at   | Thromboxane A2 receptor | TXB2R     | 0.579       | 0.0256  |
| 211385_s_at   | Sulfotransferase family, cytosolic, 1A, phenol-prefering, member 2 | SULT1A2   | 0.567       | 0.0237  |
| 217347_at     | EST        | —          | 0.563       | 0.0285  |
| 203873_at     | Possible global transcription activator SNF2L1 (SWI/SNF related matrix associated actin-dependent regulator of chromatin subfamily A member 1) | SMARCA1   | 0.557       | 0.00757 |
| 222828_at     | EST        | —          | 0.551       | 0.0493  |
| 212099_at     | Secretory carrier membrane protein 5 | SCAMP5    | 0.547       | 0.0427  |
| 213484_at     | Clone 23700 mRNA sequence | —        | 0.543       | 0.0464  |
| 209998_at     | Phosphatidylinositol glycan, class O | PIGO      | 0.499       | 0.0408  |
| 216421_at     | EST        | —          | 0.481       | 0.0194  |
| 206933_s_at   | Glucosidase, β; acid (includes glucosylceramidase) | GBA      | 0.468       | 0.0117  |
| 207740_s_at   | Nucleoporin 62 kDa | NUP62     | 0.428       | 0.0404  |
| 217142_at     | α subunit of the elongation factor-1 complex | EF1A1    | 0.416       | 0.0266  |
| 212822_at     | HEG homolog | HEG       | 0.412       | 0.0221  |
| 205777_at     | Dual specificity phosphatase 9 | DUSP9     | 0.399       | 0.0055  |
| 208533_s_at   | Ankyrin 1, erythrocyte | ANK1      | 0.205       | 0.0433  |

Fold changes and p-values of genes that changed by more than 1.7-fold in 2 μM ADMA treated HCAEC compared to untreated (p < 0.05 by Welch t-tests).

*Genes that were used for reanalysis in subsequent experiments.

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the untreated and 100-μM ADMA greater than 1.7-fold; 11 genes showed statistically significant changes at both concentrations of ADMA compared to untreated cells (Figure 2A; Tables 3 and 4).
| Affymetrix ID | Gene Title | Gene Symbol | Fold Change | p-Value |
|---------------|------------|-------------|-------------|---------|
| 222364_at     | EST        | —           | 11.06       | 0.00597 |
| 216859_x_at   | Golgi-specific brefeldin A resistance factor 1 | GBF1 | 6.757 | 0.00045 |
| 204397_at     | Echinoderm microtubule-associated protein-like | EMA2P | 4.016 | 0.0201 |
| 212417_at     | *Secretory carrier membrane protein 1 | SCAMP1 | 3.165 | 0.0307 |
| 213734_at     | Replication factor C (activator) 1, S, 36.5kDa | RFC5 | 2.740 | 0.0259 |
| 214716_at     | BMP2 inducible kinase | BMP2K | 2.695 | 0.00049 |
| 214078_at     | P21 (CDKN1A)-activated kinase 3 | PAK3 | 2.500 | 0.0228 |
| 204142_at     | Enolase superfamily member 1 | ENOSF1 | 2.488 | 0.00326 |
| 221765_at     | UDP-glucose ceramide glucosyltransferase | UGCG | 2.392 | 0.00264 |
| 215218_s_at   | Chromosome 19 open reading frame 14 | C19orf14 | 2.336 | 0.0406 |
| 213732_at     | Transcription factor 3 | TCF3 | 2.315 | 0.0141 |
| 213610_s_at   | Hypothetical protein MGC2610 | MGC2610 | 2.309 | 0.0316 |
| 201424_s_at   | Cullin 4A | CUL4A | 2.278 | 0.0356 |
| 205636_at     | SH3GL3 protein | SH3GL3 | 2.242 | 0.0395 |
| 217951_s_at   | PHD finger protein 3 | PHF3 | 2.183 | 0.0174 |
| 220112_at     | Hypothetical protein FLJ11795 | FLJ11795 | 2.128 | 0.00597 |
| 218287_at     | Chromosome 19 open reading frame 97 | C1orf97 | 2.020 | 0.0376 |
| 217540_at     | Similar to NP_055301.1 neuronal thread protein AD7c-NTP | — | 2.020 | 0.00341 |
| 222335_at     | EST        | —           | 2.016 | 0.00276 |
| 212930_at     | Hypothetical protein DNFZP64D172 | DNFZP64D172 | 1.949 | 0.0423 |
| 221649_s_at   | Peter Pan homolog (Drosophila) | PPAN | 1.989 | 0.0189 |
| 211320_at     | *Protein arginine N-methyltransferase 3 | PRMT3 | 1.883 | 0.0339 |
| 212209_at     | Thyroid hormone receptor associated protein 2 | THRA2P | 1.883 | 0.0353 |
| 204237_at     | GULP, engulfment adaptor PTB domain containing 1 | GULP1 | 1.880 | 0.0299 |
| 205191_at     | Retinitis pigmentosa 2 X-linked recessive | RP2 | 1.880 | 0.0324 |
| 202663_at     | Wiskott-Aldrich syndrome protein interacting protein | WASIP | 1.869 | 0.0233 |
| 212855_at     | KIAA0276 protein | KIAA0276 | 1.862 | 0.0192 |
| 220255_at     | Fascin anemia, complementation group E | FANCE | 1.821 | 0.0339 |
| 205296_at     | Retinoblastoma-like 1 (p107) | RB1 | 1.792 | 0.0458 |
| 206102_at     | KIAA0186 gene product | KIAA0186 | 1.786 | 0.047 |
| 200065_s_at   | Protein kinase, C, dependent, regulatory, type 1, α | PRKCA | 1.767 | 0.0491 |
| 218256_s_at   | Nucleoporin 54 kDa | NUP54 | 1.754 | 0.0223 |
| 221222_at     | Hypothetical protein FLJ20519 | FLJ20519 | 1.742 | 0.0163 |
| 221634_at     | Similar to RPL23AP7 protein | MGC70863 | 1.736 | 0.0164 |
| 201148_at     | Homeodomain interacting protein kinase 3 | HIPK3 | 1.727 | 0.037 |
| 200419_at     | Follicular lymphoma variant translocation 1 | FVT1 | 1.721 | 0.0482 |
| 217542_at     | Carboxypeptidase M | CPM | 1.701 | 0.0122 |
| 209831_x_at   | Deoxyribonuclease II, lysosomal | DNASE2 | 0.586 | 0.0186 |
| 200678_x_at   | Granulin | GRN | 0.579 | 0.0491 |
| 213829_at     | Tumor necrosis factor receptor superfamily, member 6b, decoy | TNFRSF6B | 0.577 | 0.0121 |
| 216041_x_at   | Granulin | GRN | 0.574 | 0.049 |
| 201434_at     | Tetratricopeptide repeat domain 1 | TTC1 | 0.573 | 0.0294 |
| 203778_at     | Mannosidase, α A, lysosomal | MANBA | 0.566 | 0.0395 |
| 207239_s_at   | PCTAIRE protein kinase 1 | PCTK1 | 0.565 | 0.0454 |
| 200913_at     | Protein phosphatase 1G (formerly 2C), | PPM1G | 0.564 | 0.0417 |
| 217443_at     | Zinc finger protein 592 | ZNF592 | 0.564 | 0.00276 |
| 218797_s_at   | Sirtuin (silent mating type information regulation 2 homolog) 7 | SIRT7 | 0.561 | 0.0219 |
| 200643_at     | High-density lipoprotein binding protein (vigin) | HDLBP | 0.560 | 0.0398 |
| 211941_s_at   | Prostatic binding protein | PBP | 0.556 | 0.0387 |
| 207559_s_at   | Zinc finger protein 261 | ZNF261 | 0.552 | 0.0363 |
| 214035_x_at   | LOC99491 protein | LOC99491 | 0.551 | 0.0113 |
| 216885_s_at   | WD repeat domain 42A | WDR42A | 0.551 | 0.0276 |
| 210589_s_at   | Glucosidase, I; α, acid (includes glucosylceramidase) | GBA | 0.550 | 0.0104 |
| 203124_s_at   | Mercaptopruvate sulfurtransferase | MPT5 | 0.530 | 0.0234 |
| 222217_at     | Solute carrier family 27 (fatty acid transporter), member 3 | SLC27A3 | 0.548 | 0.045 |
| 209140_x_at   | Major histocompatibility complex, class I, B | HLA-B | 0.544 | 0.0451 |
| 202581_at     | Protein expressed in nonmetastatic cells 6, (nucleoside-diphosphate kinase) | NME6 | 0.543 | 0.00934 |
| 211471_s_at   | RAB36, member RAS oncogene family | RAB36 | 0.541 | 0.0235 |
| 221757_at     | HGFL gene | MGC17330 | 0.536 | 0.0116 |
| 215533_s_at   | Ubiquitination factor E4B (UFD2 homolog, yeast) | UBE4B | 0.538 | 0.0229 |
| 215178_x_at   | EST        | —           | 0.534 | 0.0116 |
| 204248_at     | Guanine nucleotide binding protein, α-11 (Gq class) | GNA11 | 0.532 | 0.00662 |
| 212744_at     | Bardet-Biedl syndrome 4 | BBS4 | 0.528 | 0.0119 |
| 208629_at     | TAP binding protein (tapasin) | TAPBP | 0.526 | 0.0292 |
| 209002_at     | KIAA1536 protein | KIAA1536 | 0.522 | 0.0492 |
| 212400_at     | Trans-Golgi network protein 2 | TGLN2 | 0.519 | 0.0129 |
| 216283_s_at   | Poliovirus receptor | PVR | 0.519 | 0.0351 |
| 219931_s_at   | Kelch-like 12 (Drosophila) | KLEL12 | 0.510 | 0.0291 |
in HCAEC viability over 72 h in the presence of either 2 or 100 μM ADMA (Figure 2B).

Confirming Transcriptional Changes
To determine the reliability of changes identified by GeneChip analysis, four genes were selected from those that showed a statistically significant change in either the 2 or 100 μM sample compared to control (Figure 3A). These were SCAMP1, Calreticulin, (RpL27) and RpS11. In studies on a different batch of HCAEC, Northern blotting confirmed that expression of these genes changed in response to ADMA (Figure 3B).

In order to elucidate the mechanism of ADMA action, HCAEC were also treated with the potent NOS inhibitor L-NIO and SDMA, which is not a NOS inhibitor or DDAH substrate, but is a naturally occurring methylarginine that competes with arginine for the cationic amino acid transporter [10,16]. Interestingly neither SDMA nor L-NIO elicited significant changes in the expression of RpS11, RpL27, SCAMP1, or Calreticulin (Figure 3B).

| Affymetrix ID | Gene Title | Gene Symbol | Fold Change | p-Value |
|---------------|------------|-------------|-------------|---------|
| 202556_s_at   | Microsphere protein 1 | MCR51 | 0.502 | 0.00625 |
| 218216_x_at   | ADP-ribosylation-like factor 6 interacting protein 4 | ARL6IP4 | 0.619 | 0.00055 |
| 211241_at     | Lipocortin 2 pseudogene | LIP2 | 0.686 | 0.00053 |
| 202640_s_at   | RAN binding protein 3 | RANBP3 | 0.684 | 0.00046 |
| 201378_s_at   | Sjögren’s syndrome nuclear autoantigen 1 | SSNA1 | 0.680 | 0.0381 |
| 209093_s_at   | Glucosidase, β, acid (includes glucosylceramidase) | GBA | 0.667 | 0.0216 |
| 211160_x_at   | Actinin, α-1 | ADCN1 | 0.663 | 0.018 |
| 217331_at     | SCC-112 protein | SCC-112 | 0.663 | 0.0235 |
| 211911_x_at   | Major histocompatibility complex, class I, B | HLA-B | 0.444 | 0.0186 |
| 212822_at     | HEG homolog | HEG | 0.429 | 0.0275 |
| 206729_x_at   | Major histocompatibility complex, class I, B | HLA-B | 0.429 | 0.0301 |
| 210042_s_at   | Cathepsin Z | CTSZ | 0.401 | 0.0324 |
| 211230_s_at   | Phosphoinositide-3-kinase, catalytic, δ polypeptide | PIK3CD | 0.396 | 0.0428 |
| 203273_s_at   | Tumor suppressor candidate 2 | TUSC2 | 0.376 | 0.00316 |
| 218425_at     | TRAD3 protein | TRAD3 | 0.360 | 0.0426 |
| 203143_s_at   | RIAA0040 gene product | RIAA0040 | 0.354 | 0.0485 |
| 26180_s_at    | Synaptotagmin 2 | SYNU2 | 0.346 | 0.0329 |
| 206031_s_at   | Ubiquitin-specific protease 5 (isopeptidase T) | USP5 | 0.209 | 0.0292 |

| Gene Title | Gene Symbol | Fold Change | p-Value |
|------------|-------------|-------------|---------|
| PRMT3      |             |             |         |
| SDMA or L-NIO caused a small increase in PRMT3 expression (Figure 4B; n = 6).

Bone morphogenetic protein 2 inducible kinase. We identified that bone morphogenetic protein 2 inducible kinase (BMP2K) changed on the GeneChip in response to ADMA (2 μM and 100 μM ADMA increased expression by 2.304-fold [p = 0.00128] and 2.695-fold [p < 0.001], respectively; Figure 5A). In a separate series of experiments on a different batch of HCAEC this increase in BMP2K expression was confirmed by RT-PCR (data not shown). Western blotting also revealed that BMP2K protein levels were increased in response to ADMA (Figure 5B); densitometry of these blots indicated that there was a significant increase (untreated versus 2 μM, p < 0.05; and untreated versus 100 μM, p < 0.01, n = 4).

Identification of Genes Involved in Bone Morphogenetic Protein Signalling
Having confirmed changes in BMP2K expression, the total list of 765 genes that changed greater than 1.7-fold in response to 100 μM ADMA, was reexamined to identify additional genes in the bone morphogenetic protein (BMP) signalling pathway affected by ADMA. This analysis identified Smad5 and BMPRIA (Figures 6 and 7). In a separate set of studies Northern blotting confirmed that mRNA was increased in HCAEC after 24 h by either 2 or 100 μM ADMA for Smad5 and BMPRIA (Smad5 untreated versus 2 μM and untreated versus 100 μM; p < 0.05, n = 4; Figure 6A; and BMPRIA untreated versus 2 μM, p < 0.05 and untreated versus 100 μM; p < 0.01, n = 4; Figure 6B).

Identification of Global Changes in Gene Expression
All genes that changed by more than 1.7-fold (irrespective of p-value) were entered into DAVID-EASE. EASE probability scores were generated based upon the number of genes for each biological process altered in response to ADMA (Tables 5 and 6), where these biological processes were defined by...
Effects of ADMA upon Gene Expression

enriched Gene Ontology categories. These gene lists indicated that ADMA affects genes involved in metabolism, RNA splicing, transcription, and cell cycle regulation.

Gene Deletion Mice

To examine whether the effects observed in the cell culture model were relevant to the in vivo situation, we determined the expression level of certain genes in DDAH heterozygous knockout mice that have 2-fold elevation in plasma ADMA levels. Total RNA from DDAH1 heterozygous knockout was probed for mBMP2K and mPRMT3 by Northern blotting and corrected for mβ-actin expression (Figure 8). Levels of mBMP2K for brain, heart, and kidney, respectively, were 42 ± 13.8% (p = 0.047), 33.3 ± 18.6% (p = 0.038), 74.0 ± 21.4% (p = 0.007), higher in DDAH1 heterozygous mice (n = 9) compared with wild-type litter-mates (n = 5). A similar trend was seen for the expression of mPRMT3 (data not shown).

Discussion

ADMA is an endogenous inhibitor of NOSs [1] and there is an association between increased plasma levels of ADMA and renal disease [1], pulmonary hypertension [5], preeclampsia [9], and the progression of atherosclerosis [18,19]. Whilst the concentration of ADMA in plasma of healthy adults varies between 0.4 and 1 μM, it may increase to 1.45–4.0 μM with certain diseases, and this increase is thought to be causally involved in pathophysiology [1,6,7,9,20]. In the present study we detected substantial changes in gene expression in HCAEC after 24 h of exposure to concentrations of ADMA similar to those reported in pathophysiological states. Furthermore, we identified specific pathways of gene activation that give insight into the mechanisms by which ADMA may contribute to disease. Surprisingly, some of these changes appear to be independent of blockade of the L-arginine:nitric oxide (NO) pathway.

Figure 3. Confirmation of Gene Expression Changed by GeneChip Analysis

(A) SCAMP1; Calreticulin, ribosomal protein L-27 (RpL27), and RpS11 signal-to-noise ratios derived from U133A GeneChip analysis where n = 3 (untreated versus 100 μM: SCAMP1 changed 3.16-fold, p = 0.031; and untreated versus 2 μM: Calreticulin changed 2.96-fold, p = 0.047; RpS11, 8.065-fold, p = 0.033; RpL27, 4.39-fold, p = 0.048).

(B) SCAMP1; Calreticulin, RpL27, and RpS11 mRNA levels are elevated by ADMA (2 and 100 μM; *p < 0.05 and **p < 0.01). SDMA (100 μM) and L-NIO (100 μM) did not elicit changes in gene expression as shown by Northern blotting, where mRNA was corrected for differences in β-actin mRNA expression. DOI: 10.1371/journal.pmed.0020264.g003
Low Concentrations of ADMA Alter Gene Expression

Acute administration of ADMA to healthy individuals elicits a transient fall in heart rate and cardiac output and increases blood pressure [2], but little is known of the potential longer-term effects of raised ADMA. Zoccali et al. reported that a 1-µM increment in ADMA above the upper limits for healthy individuals was associated with increased risk of cardiovascular mortality [7], and levels around 2 µM seem to be associated with a number of diverse cardiovascular pathologies [3,8]. In the current study, HCAEC were treated with 2 or 100 µM ADMA. We repeated GeneChip analysis in three separate studies and observed reproducible changes in the expression of a subset of genes in response to low- and high-dose ADMA. Because there is always a possibility of false positives being identified on arrays, we tested the reproducibility of the GeneChip approach by selecting four genes significantly upregulated by ADMA treatment. In a separate series of studies we confirmed an increase in mRNA levels by Northern blotting at both concentrations of ADMA. Thus it is clear that pathophysiological concentrations of ADMA (2 µM) affect endothelial cell gene expression even in the presence of very high arginine concentrations (>300 µM). Endogenous ADMA is metabolised by DDAH, and DDAH activity is the major determinant of plasma ADMA concentrations [2]. To determine whether the effects we saw in vitro would be reproduced in vivo we examined DDAH1 knockout mice. At least one of the gene changes we saw in vitro also occurs in vivo since DDAH1 heterozygous knockout mice have increased concentrations of ADMA in plasma and showed upregulation of BMP2K in several tissues. ADMA inhibits NOSs with an IC50 of about 5 µM, the precise potency depending on the prevailing concentration.

Figure 4. ADMA Alters PRMT3 Gene Expression and Protein Levels
(A) PRMT3 levels are changed by ADMA on U133A GeneChips where n = 3 (untreated versus 100 µM: 1.88-fold increase, p = 0.034).
(B) PRMT3 mRNA is changed in HCAEC following 24-h exposure to ADMA (2 and 100 µM) but not SDMA (100 µM) or L-NIO (100 µM), measured by Q-PCR (p < 0.05 and **p < 0.01, where n = 8).
(C) PRMT3 protein levels are increased in HCAEC following 24-h treatment with ADMA (2 and 100 µM) as determined by Western blotting, using a commercially available PRMT3 antibody (Upstate Biotechnology), where equal amounts of protein were loaded in each lane. Densitometry of the PRMT3 was carried for each of the blots from four separate experiments. ADMA (2 and 100 µM) treatment for 24 h significantly increased the levels of PRMT3 (p < 0.05). The inset blot is a representative from four separate experiments. DOI: 10.1371/journal.pmed.0020264.g004

Figure 5. ADMA Alters BMP2K Gene Expression and Protein Levels
(A) BMP2K is increased more than 2-fold in HCAEC following 24-h ADMA treatment (2 µM and 100 µM ADMA increased expression by 2.304-fold [p = 0.00128] and 2.695-fold [p < 0.001] respectively).
(B) BMP2K protein levels are increased in HCAEC following 24-h treatment with ADMA (2 and 100 µM) as determined by Western blotting, using a commercially available BMP2K antibody (Orbigen). Densitometry of the BMP2K band was carried for each of the blots from four separate experiments (untreated versus 2 µM: p < 0.05; and untreated versus 100 µM: p < 0.01, n = 4). The inset blot is representative of four separate experiments, where equal amounts of protein were loaded in each lane. DOI: 10.1371/journal.pmed.0020264.g005
It is known that adding endogenous NO to endothelial cells alters gene expression and that inhibitors of endogenous NO generation can alter expression of specific genes, at least under conditions of endothelial cell activation with cytokines. To determine whether the effects we observed could be accounted for by inhibition of NOS, we treated cells with a highly potent NOS inhibitor (L-NIO) and with SDMA, an endogenous dimethylarginine that has no effect on NOS but which can block arginine transport. Neither SDMA nor L-NIO replicated the effects of ADMA on gene expression. We have not undertaken a full GeneChip analysis of responses to L-NIO, so we do not know how much overlap there would be between the effects of L-NIO and ADMA, but of those genes examined we saw a discordance between responses to the two inhibitors. This raises the intriguing possibility that some of the actions of ADMA may be independent of effects of NO, possibly due to other actions such as the ability of ADMA to increase superoxide generation.

Patterns of Gene Change

Mapping genes changed by ADMA to identify global changes in biological processes, indicated that ADMA treatment may have significant effects on genes involved in cell cycle regulation, cell proliferation, DNA repair, transcriptional regulation, and metabolism. The full biological significance of the range of genes affected is not yet known, but our data demonstrate the potential for elevated ADMA to affect endothelial (and likely other cellular) function in disease. In the present study, we focussed on two pathways of potential importance—BMP pathways and PRMTs.

BMP Pathways and ADMA

Analysis of the U133A GeneChips revealed that BMP2K was induced more than 2-fold in response to either 2 or 100 μM ADMA. The increase in gene expression was mirrored by an increase in BMP2K protein, and the effect was also seen in our high-ADMA mouse model. By relaxing parameters (to exclude false negatives) a search for other genes involved in BMP signalling revealed that Smad5 and BMPR1A were amongst the transcripts increased by GeneChip analysis, and these changes were confirmed by Northern blotting. The finding of changes in the BMP signalling pathway is important since the ADMA/DDAH pathway seems to be involved in animal models of pulmonary hypertension, and mutations in the BMP receptor 2 (BMPR2) are associated with familial pulmonary hypertension in humans.

The changes in BMP pathways may also be important in understanding some of the effects of renal failure. ADMA accumulates in renal failure and fulfils many of the criteria of a uraemic toxin. In addition to effects on cardiovas-
cullar risk, ADMA may contribute to renal osteodystrophy, a process in which BMPs have been implicated [31]. Indeed an earlier study that showed that ADMA reduces osteoblast process in which BMPs have been implicated [31].

The present study confirms osteocalcin as a gene downregulated following exposure to ADMA [32]. Amongst the genes changed greater than 1.7-fold in response to ADMA was BMP2K (Figure 7). Whatever the mechanisms, identification of a link between ADMA and BMP pathways may be relevant to the increased vascular calcification seen in renal disease [31].

ADMA and Arginine Methylation

We observed that PRMT3 gene expression was elevated following exposure to ADMA; this was confirmed by Q-PCR, and PRMT3 protein expression also increased. This is the first report that ADMA may alter the expression of enzymes involved in its own synthesis. There are presently five known PRMTs that asymmetrically methylate arginine residues and two (PRMT5 and PRMT7) that symmetrically methylate arginine residues. PRMT3 has a wide tissue distribution, is expressed in highly vascular tissues, including heart and lung [17], and expression may be increased by oxidised-LDL [34]. Our observations indicate that ADMA can induce a similar increase in PRMT3 expression.

The roles of methylation of arginine residues in proteins are not yet well defined, but studies of PRMT3 in fission yeast have shown that it associates with proteins involved in the translational machinery and that the S2 ribosomal protein, a component of the yeast 40S ribosome, is a specific substrate for PRMT3 [35]. PRMT3 is the only PRMT known to interact with the translational machinery, and it is interesting that we have found several genes, including ribosomal proteins RpS11 and RpL27, involved in translational control, that were also altered in response to ADMA treatment. Amongst the genes changed greater than 1.7-fold in response to ADMA was methionine adenosyltransferase II α, which catalyses the production of S-adenosylmethionine, the methyl donor for the PRMT reaction [36]. The role of ADMA in regulating arginine methylation in protein deserves further study.

Summary

Increased circulating concentrations of ADMA have been reported in cardiovascular and other disorders, and intracellular concentrations may vary independently of circulating levels. In the present study we have demonstrated that relatively small changes in the concentration of ADMA affect gene expression in endothelial cells. Identification of pathways regulated by ADMA may aid our understanding of how ADMA contributes to a wide range of pathologies. Two pathways of specific interest have been identified—BMP signalling and enzymes involved in arginine methylation. The effects on BMP signalling may be particularly important in renal disease and in the link between raised ADMA and pulmonary hypertension.

Supporting Information

Accession Numbers

The microarray data have been loaded into the EBI MIAExpress database (http://www.ebi.ac.uk/miaexpress/) and have been assigned the accession number E-MEXP-377.
Effects of ADMA upon Gene Expression

Figure 8. BMP2K Is Increased in DDAH1 Gene Deletion Mice
Expression of BMP2K mRNA from brain, heart, and kidney is increased in 12-wk DDAH1 heterozygous knockout mice compared to wild-type littermates (p = 0.0473, p = 0.0379, and p = 0.0070, respectively); mRNA was corrected for differences in β-actin mRNA expression, where n = 5 wild type, and n = 9 DDAH heterozygous.

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Patient Summary

Background Diseases of the circulation system are common and cause many deaths. Medical conditions associated with damage to the blood vessels include heart failure, high blood pressure, stroke, and kidney failure. The lining of the blood vessels plays an active role in maintaining their health. A substance called asymmetric dimethylarginine (ADMA) is found naturally in the vessel lining, both in healthy people and in people with vascular disease, but in the latter it is present at higher levels. Thus raised ADMA may be a marker of vascular disease. This means it could be used to help identify people with a circulation problem. However, it has not been clear whether ADMA actually causes any damage, i.e., whether it is more than just a marker.

What Did the Researchers Do and Find? The researchers are trying to find out whether elevated ADMA levels can cause vascular disease. In this study, they treated cells from blood vessel linings with levels of ADMA equal to those found in people with vascular disease and measured how gene activity changed in response. They found that a number of genes were more active when the cells were exposed to the elevated ADMA levels. Some of these were interesting because other studies suggest that they might be involved in lung, heart, and kidney disease.

What Do the Results Mean for Patients? This area of research is still at an exploratory stage. Additional studies need to examine which function (if any) the genes that respond to elevated ADMA levels play in vascular disease. If they do play active roles, drugs that inhibit them might help to prevent or treat vascular disease.

Where Can I Get More Information? For general information on cardiovascular disease see information provided by the following organisations.

The British Heart Foundation: http://www.bhf.org.uk/hearthealth/index_home.asp?SecID=1

The American Academy of Family Physicians: http://familydoctor.org/292.xml

The National Heart, Lung, and Blood Institute: http://www.nhlbi.nih.gov/health/public/heart/index.htm

New York Online Access to Health: http://www.noah-health.org/en/blood/vascular/index.html

University College London: http://www.ucl.ac.uk/medicine/clinical-pharmaco/research