Sodium nitrite protects against kidney injury induced by brain death and improves post-transplant function

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Renal injury induced by brain death is characterized by ischemia and inflammation, and limiting it is a therapeutic goal that could improve outcomes in kidney transplantation. Brain death resulted in decreased circulating nitrite levels and increased infiltrating inflammatory cell infiltration into the kidney. Since nitrite stimulates nitric oxide signaling in ischemic tissues, we tested whether nitrite therapy was beneficial in a rat model of brain death followed by kidney transplantation. Nitrite, administered over 2 h of brain death, blunted the increased inflammation without affecting brain death–induced alterations in hemodynamics. Kidneys were transplanted after 2 h of brain death and renal function followed over 7 days. Allografts collected from nitrite-treated brain-dead rats showed significant improvement in function over the first 2 to 4 days after transplantation compared with untreated brain-dead animals. Gene microarray analysis after 2 h of brain death without or with nitrite therapy showed that the latter significantly altered the expression of about 400 genes. Ingenuity Pathway Analysis indicated that multiple signaling pathways were affected by nitrite, including those related to hypoxia, transcription, and genes related to humoral immune responses. Thus, nitrite therapy attenuates brain death–induced renal injury by regulating responses to ischemia and inflammation, ultimately leading to better post-transplant kidney function.

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Brain-dead (BD) donors account for a significant source (~60%) for all renal allografts transplanted in the United States. Kidneys acquired from BD donors have inferior survival rates compared with living donors because of brain death–induced organ injury that comprises initially of massive acute cerebral injury, neurogenic shock, systemic vasoconstriction, and hypoperfusion of multiple organs including the kidney.[1–4] In addition to the tissue ischemia that ensues, electrolyte abnormalities and increased levels of pro-inflammatory cytokines after brain death combine to result in inflammatory injury to the kidney, which primes this organ for an exacerbation of ischemia–reperfusion (I/R) injury response after transplantation.[5,6] This 'two-hit' process is a major contributor to poor kidney function and allograft survival after transplantation using organs from BD donors and has been demonstrated with other organ transplants. Limiting ischemic and inflammatory injury by treating the BD donor with corticosteroids, recombinant soluble P-selectin glycoprotein ligand, or erythropoietin has been shown to improve post-transplantation renal function and survival in experimental models, supporting the idea that therapeutics targeting the BD phase can be beneficial.[7–9]

Nitric oxide (NO) is an important endogenous modulator of inflammation, and at lower concentrations it protects against inflammatory stress by multiple mechanisms including limiting leukocyte adhesion, antioxidant activity, improving tissue oxygenation, and inhibiting cell death. This view is supported by studies showing that therapies that increase NO bioavailability confer protection against I/R injury in both experimental and clinical studies.[10–17] Major limitations with NO therapy are unwanted systemic vasodilator effects, highlighting the lack of therapies that target NO formation to specific tissues.

Inorganic nitrite (NO_2^-) has long been considered an inert end product of aerobic NO metabolism. However, recent insights reveal the presence of biological mechanisms that result in the 1-electron reduction of nitrite back to NO and stimulation of NO-dependent signaling. Importantly, this reduction is focused in hypoxic (or ischemic)/acidic tissues resulting in targeted NO formation. Proposed biochemical mechanisms include nitrite reduction by heme/molybdenum-containing metalloproteins (including xanthine oxidoreductase, hemoglobin, and myoglobin), whose ability to reduce nitrite is dependent on lower oxygen tensions.[18–22] Consistent with this concept, several studies have shown the potential for nitrite therapy to stimulate NO signaling only in ischemic tissues and mitigation of I/R injury.[11,13,23–28] Moreover, there is precedent for NO-based therapy to counter the detrimental effects of brain death. L-arginine therapy repletes NO signaling and protects against brain death–dependent endothelial dysfunction and myocardial blood flow.[29]

In this study, we evaluated the potential of nitrite to protect against I/R damage that occurs to the kidney during brain death and improve function after transplantation. Presented data show that nitrite therapy to the BD donor can be administered safely and can improve function after transplantation via anti-inflammatory mechanisms.

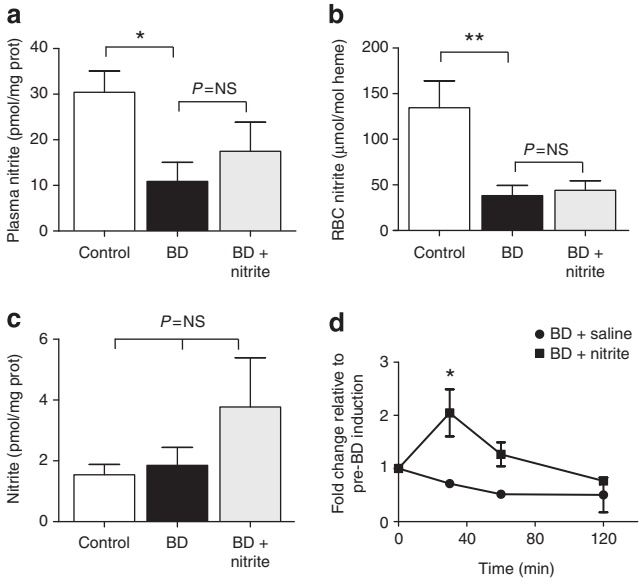

Figure 1 | Circulating nitrite levels are decreased during brain death (BD). (**a**) Plasma, (**b**) red blood cell (RBC), and (**c**) kidney concentrations of nitrite were measured in rats after 2 h of sham (control), BD, or BD + nitrite therapy. Data show means ± s.e.m., $n = 3$–5. *$P < 0.05$, **$P < 0.02$ relative to control by *t*-test. NS, not significant. (**d**) Time-dependent changes in plasma nitrite levels in BD and BD ± nitrite therapy groups. Data show fold change relative to time 0 (before BD induction) and are mean ± s.e.m. ($n = 2$–3). $P < 0.01$ by two-way analysis of variance (ANOVA) and *$P < 0.01$ by Bonferroni post test. Plasma nitrite levels without normalization to protein ranged between 44 and 1140 nM.

RESULTS

Brain death (2 h) decreases circulating, but not renal, nitrite levels

Nitrite levels decrease during ischemic stress and nitrite therapy has been shown to improve renal function after I/R.[13,25,28,30] Using nitrite + nitrate as an index for NO production, previous studies have concluded that excessive NO formation does not occur during 2 h of brain death in rats.[31] Recent studies, however, have suggested that nitrite is a more selective index of NO formation in mammals.[32] Figure 1 shows that circulating nitrite levels are decreased ~60–70% after 2 h of brain death. However, renal nitrite levels did not change after brain death.

Hemodynamic effects of nitrite therapy during brain death

During induction of brain death in this model, mean arterial pressure (MAP) increases and then rapidly (within 10 min) decreases and remains lower than that before brain death (Figure 2). Nitrite is a vasodilator and can promote hypotension. Initial studies were therefore designed to determine the optimal conditions for nitrite administration that would not exacerbate hypotension during brain death. Bolus administration of nitrite (0.1 mg/kg) led to a lower MAP compared with brain death alone (not shown). We therefore tested an alternative nitrite administration protocol in which nitrite (0.1 mg) was administered over 2 h of brain death. Figure 2 shows that using this protocol nitrite did not alter MAP compared with brain death alone and was tested further for potential protective effects against brain death–induced kidney injury.

Nitrite therapy improves post-transplantation function of kidneys after brain death

Kidneys from rats rendered BD for 2 h, which were administered either saline or nitrite, were transplanted and renal function assessed by measuring plasma creatinine and

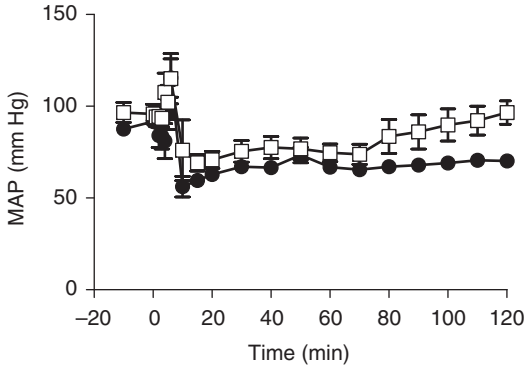

Figure 2 | Effects of nitrite therapy on mean arterial pressure (MAP) during brain death (BD). Rats were rendered brain dead (-●-) and then administered nitrite (0.1 mg/kg) over 2 h (-□-). No significant difference between BD alone or BD + nitrite administered over 2 h by two-way repeated measures analysis of variance (RM ANOVA) was observed. Data show mean ± s.e.m. ($n = 5$) for BD alone or BD + continuous infusion of nitrite.

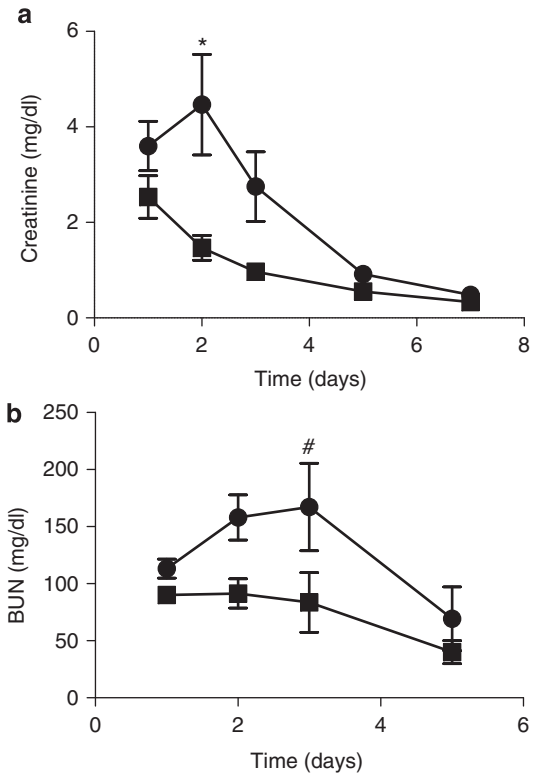

Figure 3 | Nitrite treatment improves post-transplantation renal function after brain death (BD). Kidneys were isolated from brain-dead rats treated with saline (-●-) or nitrite (-■-) and then transplanted. Data show (**a**) serum creatinine and (**b**) blood urea nitrogen (BUN) levels in the recipient as a function of time after transplantation. Data are means ± s.e.m. ($n = 6$) and are significantly different ($P < 0.0001$ for creatinine and $P < 0.005$ for BUN) by two-way analysis of variance (ANOVA) and $^{*}P < 0.001$ or $^{\#}P < 0.05$ by Bonferroni post test relative to BD + nitrite.

blood urea nitrogen (BUN) as a function of time after transplantation. Figure 3 shows that after transplantation of a kidney after brain death, plasma creatinine and BUN increased for 2–3 days and then started to decrease, indicating an initial worsening of renal function followed by resolution of injury. After transplantation of kidneys isolated from BD rats treated with nitrite, however, creatinine and BUN levels were lower and decreased at a faster rate compared with the BD-alone group. Figures 1a–c shows that the protective effects of nitrite occurred without significant increased nitrite levels in the plasma, red blood cells, or kidneys 2 h after brain death induction. However, Figure 1d shows that although circulating nitrite levels decrease from the onset of brain death alone, with nitrite therapy plasma nitrite levels are elevated for the initial 30–60 min and then gradually decrease over the next 60 min.

Nitrite prevents brain death–induced inflammation in the kidney but not lipid peroxidation

To evaluate the mechanisms underlying improved renal function after transplantation of kidneys collected from nitrite-treated BD rats, expression of several markers/mediators of inflammation and oxidative stress were measured in kidneys collected after 2 h of brain death. Figure 4 shows representative immunofluorescence staining for the adhesion molecules CD11a, CD11b, CD18, and the protein adduct of 3-hydroxy nonenal. All these markers increased after 2 h of brain death and showed a tubular location consistent with increased inflammation and oxidative stress. Nitrite treatment decreased staining for CD11a and CD11b, which was further confirmed by a significant decrease in CD11b measured by western blotting (Figure 4b). Interestingly, nitrite did not affect 3-hydroxy nonenal staining. No changes in the expression of the pro-inflammatory peroxidase myeloperoxidase, the inflammatory marker 3-nitrotyrosine, or adhesion molecules intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 were observed in BD-exposed rats (not shown).

Effect of 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-l-oxyl-3-oxide (C-PTIO) and allopurinol on nitrite-dependent protection against renal inflammation

Previous studies have shown that NO scavenging (by C-PTIO) and/or inhibition of nitrite reduction to NO by xanthine oxidoreductase (using allopurinol) prevents nitrite-dependent protection against ischemic tissue toxicity. Using CD11b expression as a marker, C-PTIO had no effect on brain death–induced renal injury and did not significantly reverse the protective effects of nitrite, although a trend toward a reversal is noted (Figure 4b). Allopurinol itself protected against brain death–induced renal inflammation. Nitrite had no additional effects and allopurinol did not reverse nitrite-dependent inhibition of CD11b expression (Figure 4b).

Nitrite-dependent changes in gene expression determined by microarray profiling

Supplementary Figure S1A online shows a heat map showing that nitrite therapy altered the expression of 381 genes relative to brain death alone, with 264 being downregulated and 117 upregulated. Ingenuity Pathway Analysis (IPA) generated 19 networks (with >1 focus molecule identified) affected by nitrite therapy (Table 1), and was consistent with recent reports showing that nitrite therapy has diverse effects on gene expression profiles.[33] Interestingly, the highest ranked network was related to the humoral immune response, an established key element of inflammatory tissue injury during brain death in the kidney.[34] Supplementary Figure S2 online illustrates the top five canonical signaling pathways that are most significantly affected by nitrite therapy (elF2, Notch, HIF1α, Nrf2/oxidative stress, and elF4/p70S6K, respectively) and also indicates specific genes that may be modulated by nitrite therapy. Supplementary Figure S1B online lists the number of genes that are downregulated or upregulated within these pathways. Figure 5 shows real-time PCR analyses of nine selected genes encompassing the top five pathways and separates these into those that either did not change or were upregulated by nitrite plus brain death relative to brain death alone. Supplementary Table S1 online compares mRNA array data with real-time PCR data and shows that nitrite-dependent changes indicated by mRNA arrays were validated

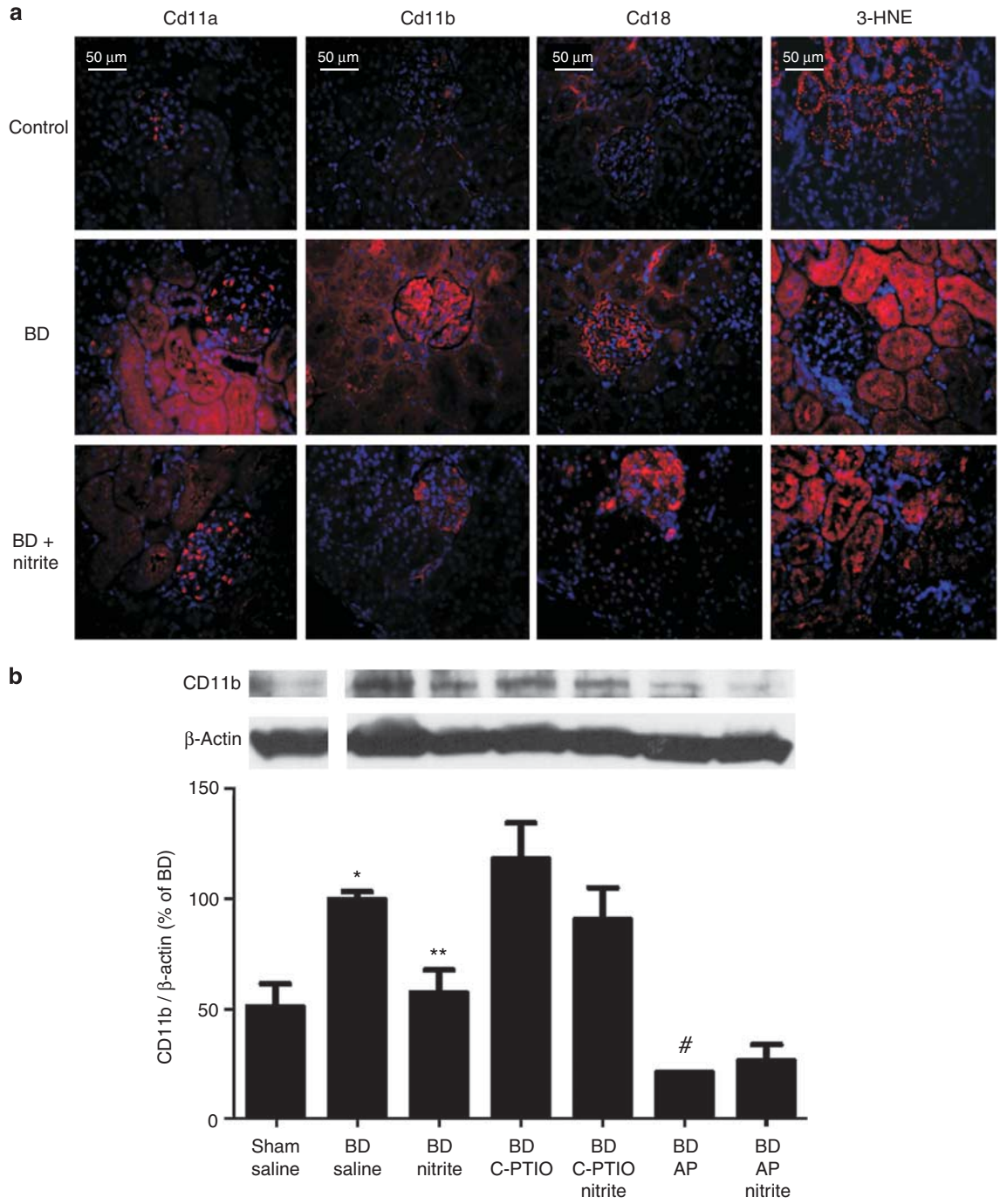

Figure 4 | Nitrite treatment prevents brain death (BD)–induced increases in markers of leukocyte infiltration but not oxidative stress. (**a**) Representative immunofluorescence images for Cd11a, Cd11b, Cd18, and 3-HNE (red staining) in control, BD (2 h), or BD (2 h) + nitrite groups. Blue staining represents nuclei stained with Hoechst 33342. (**b**) Western blot analysis for expression of CD11b/β-actin ratios. Data show mean ± s.e.m. ($n = 2$–6) and are normalized to the BD-alone group (set at 100%). $P < 0.001$ by one-way analysis of variance (ANOVA) with $^{*}P < 0.05$ relative to sham, $^{**}P < 0.05$ relative to BD alone, $^{\#}P < 0.01$ relative to BD alone by Tukey's multiple comparison post test. C-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-l-oxyl-3-oxide; 3-HNE, 3-hydroxy nonenal.

with four of the selected genes. Finally, as one of the genes validated to increase with nitrite therapy was Keap1, relative expression of Nrf2 was also determined. Figure 5 shows that this was increased as well with nitrite therapy relative to brain death alone.

DISCUSSION

BD donors comprise a significant resource of kidney allografts for transplantation. A major limitation of organs collected from BD donors is the enhanced sensitivity of the organ to injury after transplantation when compared with

Table 1 | Signaling networks and components affected by nitrite therapy as identified by Ingenuity Pathway Analysis (IPA)

ID	Molecules in network	Score	Focus molecules	Top functions
1	↓**ADAM17**, ↑**ANXA13**, Cbp, ↑**COPS2**, ↑**COPS3**, ↑**DUSP5**, ↑**ECSIT**, ↑**HLA-C**, ↑**HLA-G**, IFN-α/β, IFN-β, ↑**IRF3**, ↑**LITAF**, ↑**LRRFIP1**, ↑**LY6C1**, MHC Class I (complex), MHC CLASS I (family), NF-κB (complex), ↑**NLRC4**, Notch, ↑**NUP98**, ↓**NUPL1**, ↑**PAK1IP1**, ↓**PDIA3**, ↓**PNPT1**, ↑**PTMA**, ↑**S100A6**, ↑**SEC13**, ↑**SLC2A5**, sphingomyelinase, ↑**SUGT1**, Tap, ↓**TFG**, ↓**TFPI**, ↑**ZMYND11**	43	26	Antigen presentation, antimicrobial response, humoral immune response
2	Akt, Ap1, ↑**APEX1**, ↑**ASCC2**, ↓**ASS1**, Ck2, ↑**CPT1B**, Cyclin A, ↓**DARS**, ↑**DGCR6**, ↑**DHRSX**, ↓**DNMT1**, ↓**EED**, ↑**EEF1D**, ↑**EGLN2**, Estrogen receptor, ↑**HBB (includes EG:3043)**, ↓**JUN**, ↑**KLF7**, ↓**M6PR**, ↓**NFKBIB**, oxidoreductase, ↑**PDCD2**, peptidase, ↑**PI4KA**, ↑**PIP4K2A**, ↓**PSAP**, Rar, ↓**RORA**, Rxr, ↑**SLC19A1**, ↓**SNW1**, ↑**THRB**, Thyroid hormone receptor, ↑**VARS**	42	25	Gene expression, tissue morphology, amino acid metabolism
3	α-Tubulin, ↑**BNIP3L**, ↑**CCT5**, Creb, ↑**DCN**, ↑**DUSP3**, ↑**EIF2B4**, ERK1/2, FSH, ↓**GEM**, Gsk3, ↑**GSK3B**, ↑**HSPA14**, Insulin, ↑**KLC1**, Lh, ↑**MAP4**, p70 S6k, ↑**PAF1**, ↓**PAH**, PDGF BB, ↑**PDPK1**, Pi3-kinase, ↓**POLR2B**, PP1 protein complex group, PP2A, Ppp2c, ↑**PPP2R2D**, ↓**RBBP5**, ↑**ROCK2**, Rsk, ↑**SLC6A6**, ↑**STIP1**, ↓**STK16**, ↑**TXK**	33	21	Cell morphology, reproductive system development and function, developmental disorder
4	↑**C4ORF27**, CDC5L, ↑**COMMD9**, ↑**COQ6**, ↑**GLTSCR2**, HNF1A, HNF4A, ITGB3BP, ↓**KIAA1012**, ↑**MRPL15**, ↑**MRPL22**, ↑**MRPS11**, ↓**MRPS14**, ↓**MTHFS**, POLRMT, ↑**PRCC**, SFRS2, ↓**SLC35A3**, ↑**TFB2M**, TGFB1, ↑**TMEM140***, ↑**TTC22**, ↑**ZNF410**	27	16	Gene expression, tissue development, endocrine system development and function
5	Alp, α-catenin, ↑**ATF4**, Caspase, ↑**COL4A3**, Collagen type I, Collagen type IV, ↑**DDX31**, ↑**DDX52**, ↑**EDNRB**, ↑**HBXIP**, ↓**HES1**, Holo RNA polymerase II, Hsp70, IL1, IL12 (complex), Immunoglobulin, Jnk, LDL, ↑**MED29**, ↓**MED30**, Mmp, ↓**NCSTN**, ↓**NME3**, ↑**NOP58**, PI3K, ↑**PSEN1**, ↑**PTGES**, RNA polymerase II, ↑**TAF8**, ↑**TARDBP**, ↑**TCEB1**, Tgf-β, ↓**TMED2**, Vegf	26	18	Nervous system development and function, organ development, embryonic development
6	ABCD3, ↑**AP3B1**, ↑**BAT1**, ↑**BLOC1S1**, BNIP1, ↑**EMG1**, ↑**HDDC3**, HNF4A, LSM3, ↑**LSM4**, LSM5, ↓**LUC7L2**, ↑**MRPL43**, NAPA, NMI, NSF, ↑**PELO**, ↑**POLD4**, POLE, POLL, ↑**PPP6C**, RFC3, SART3, SEC22B, ↑**SFRS11**, ↑**SLC25A38**, SOX10, ↑**STK19**, STX18, SUPT7L, TADA1, ↑**TEAD3**, ↑**TMEM87B**, ↑**USE1**, ZSCAN16	25	17	Cellular assembly and organization, molecular transport, protein trafficking
7	↑**B3GNT1**, ↑**BANP**, ↑**BMPR2**, Calcineurin protein(s), Cdc2, ↑**EPS8**, ↑**EPS8L2**, ERK, F Actin, ↑**FLNB**, Focal adhesion kinase, ↑**HABP4**, hCG, Ige, ↓**IRX1**, ↓**JUNB**, ↓**LUC7L2**, ↑**MAP3K4**, ↑**MAPK14**, ↑**MAPKAPK2**, Nfat (family), NGF, ↑**NOP56**, P38 MAPK, Pdgf, Pka, Pkc(s), ↑**PLCG1**, Rac, Ras, ↑**RPA2**, ↑**RYR1**, Sapk, TCR, VAV	21	17	Cell morphology, cellular assembly and organization, genetic disorder
8	↓**C10ORF2**, DBN1, DDX56, ↑**EG623169**, EIF4A2, glutamyl-Se-methylselenocysteine, JRK, ↑**LOC440733**, LONP1, LYAR, MAGEB2, ↑**MRPL23**, MT-ND4, MT-ND5, MT-ND6, MT-ND4L, ↑**MYO1C**, ↑**NDUFA10 (includes EG:678759)**, NDUFV2, PA2G4, ↓**PGCP**, phosphatidylinositol-3-phosphate, PIN4, PINX1, ↑**RPL24**, ↑**RPL10A (includes EG:19896)**, ↑**RPL13A**, ↑**RPL18A**, ↑**RPS11**, selenomethylselenocysteine, SLC2A4, SNX1, ↑**TXNDC12**, ↑**WDFY1***, WDR8 (includes EG:49856)	19	14	Cancer, genetic disorder, neurological disease
9	ANAPC13, ↑**BAT1**, ↓**C16ORF80**, ↑**CDC16**, CDC27, ↑**DNAJC11**, ↑**DPEP1**, DUSP15, EXTL3, ↑**FAM110A**, ↑**FNBP4**, FYN, GRB2, HTT, ISG20L2, ↓**LUC7L2**, MEPE, ↑**OSBPL3**, PAX3, ↑**PDE10A**, POP1, POP4, ↑**RBM25 (includes EG:58517)**, RIN3, ↑**RPP14**, RPP21, RPP30, RPP38, RPP40, ↑**RPS13**, SAPS3, SUV39H2, SVIL, ↑**TMEM102**, YWHAG	19	14	Nervous system development and function, organismal development, neurological disease
10	AHSG, ANAPC2, ANAPC4, ↑**ATOX1**, BACE1, BMP1, ↑**DAZAP1**, ↑**DCN**, EIF4E, ↑**ENTPD5**, Eotaxin, Ferritin, FN1, ↑**GNL1**, ↑**HCCS**, Ifn, ITGB6, LGALS3BP, NAIP, ↑**PAPPA**, ↑**PDRG1**, PGF, PRG2, ↑**PTMA**, ↑**SGCA**, ↑**SNRPA**, SOCS, SOCS3, ↓**ST6GAL1**, STX6, SVIL, Talin, TNF, ↑**VPS45**, XIAP	17	13	Inflammatory disease, skeletal and muscular disorders, cellular response to therapeutics
11	↓**AASS**, ↑**ACVR2B**, AHNAK, ↑**BANP**, BUB1, CDH13, CDKN1A, ↑**CNIH4**, ↑**CNKSR3**, COL6A1, COL6A3, DHX36, ERBB2, ↑**FAM110B**, ↑**GALNT3**, ↓**GOLGA5**, IARS, ILK, LXN, ↑**MAP4**, MSTN, NET1, ↓**PANK2**, PARVA, RAB1A, Rb, ↑**RFC4**, RRAD, ↑**S100A6**, SPINT2, TGFB1, Type II Receptor, VPS39, YWHAZ, ↑**ZNF593**	17	13	Genetic disorder, skeletal and muscular disorders, skeletal and muscular system development and function
12	ATXN1, β-estradiol, ↑**C13ORF33**, ↑**CDK11A**, EDA, EIF3, EIF3B, EIF3CL, ↑**EIF3D**, EIF3G, ↑**EIF3H**, EIF3K, EIF3L, ↓**EIF4G3**, GAR1, GARS, GMFB, ↓**KIAA2026**, MBP, NARS, ↑**NHP2**, PNP, PTPRD, ↑**PYGM**, ↓**R3HDM2**, RCN2, RHPN2, ↑**SAMD8**, SAPS2, ↑**SPOCK2**, TGFBR1, TRAF6, VAMP1, ↓**VAPA**, ↑**WFDC2**	17	13	Protein synthesis, digestive system development and function, cell death
13	ASCC3, ↓**AUH**, BATF, ↑**C21ORF7**, C3AR1, ↓**C9ORF46**, ↑**CD248**, CD3EAP, COL6A3, CYSLTR2, ↑**DUSP11**, GPX4, ↑**HDLBP**, IL4, IL13, IL15, IL19, ↑**LAS1L**, LGALS3BP, LSP1, MIR373, ↓**MKRN1**, ↓**MRPS10**, RELA, RETNLA, ↓**RNF25**, SFRS7, ↓**SLC25A23**, SPINT2, SPRR1A, ↑**ST7**, ↓**SYAP1**, TMEM9B, TNFSF4, XRCC6	17	13	Cellular growth and proliferation, tissue development, cell-to-cell signaling and interaction
14	↑**DUSP5**, FKBP1A, FKBP1B, GATA3, ↓**GOLGA7**, HRAS, IL5, ↑**KCTD9**, KLF3, MIR302A (includes EG:407028), ↓**MYADM**, peptidylprolyl isomerase, PIN4, PPIB, PPID, PPIF, ↑**PPIL3**, ↑**RPS10**, ↑**SCYL1**, SERPIND1, ↑**SLU7**, SRF, ↑**ST7**, TIMM10, TIMM17B, ↑**TIMM8B**, TOMM6, TOMM7, TOMM20L (includes EG:75266), TOMM40L, TOMM5 (includes EG:68512), ↓**UPB1**, ↓**ZC3H8**, ZDHHC9, ↑**ZNF207**	17	13	Post-translational modification, protein folding, cancer
15	↑**ACE2**, AMPK, ↓**ANGEL1**, CHAF1A, CXCL1, ↑**DCN**, ↑**DDX24**, DPM2, DUSP1, ↑**HDAC1L**, HEXIM1, IL6, KIF11, MAP3K1, ↓**MLF2**, NOP2, phosphatidylinositol *N*-acetylglucosaminyltransferase, PIGA, PIGB, PIGC, ↓**PIGH**, PIGP, PIGQ, PIGY, RNase A,	17	13	Tumor morphology, cellular development, hematological system development and function

Table 1 Continued on the following page

Table 1 | Continued

ID	Molecules in network	Score	Focus molecules	Top functions
	↑**RPL10A (includes EG:4736)**, ↓**SALL1**, ↑**SP110**, SPTAN1, TMEM9B, ↓**TSSC4**, VEGFA, ↓**YIPF4**, ZFP36, ↑**ZNF692**			
16	APH1A (includes EG:51107), APH1B, ↓**BCAS2**, CAPN1, CCAR1, COMT, DDIT4, ↓**ECHS1**, ↓**EI24**, GLUL, ↓**JMJD8**, JMJD1C, LIG3, MIR34A (includes EG:407040), ↑**NLRC4**, NR3C1, PARP, ↓**PARP2**, ↓**PARP4**, ↑**PHF17**, ↑**PLEKHF1**, PSENEN, ↑**RBBP6 (includes EG:5930)**, ↓**SCAMP1**, SNCAIP, SUPT16H, TEP1, TP53, TPT1 (includes EG:7178), Ube3, ↑**UFC1**, ↓**UFSP2**, VHL, XRCC1	16	13	Post-translational modification, cell death, small molecule biochemistry
17	ACTR6, AGT, AGTR1B, ARPC5, FOLR2, FOS, GNAO1, Immunoproteasome Pa28/20s, ↑**LOC119358***, MOS, ↑**MSLN**, POMP, ↓**PRELID1**, Proteasome PA700/20s, PSMA, PSMA1, PSMA4, PSMA5, ↑**PSMA7**, PSMB2, PSMB3, PSMB4, ↑**PSMB6**, ↑**PUS1**, RARB, ↑**RGS11**, RPL7A (includes EG:6130), ↑**SLC4A3**, SNCG, STAT6, TOM1, ↑**TWISTNB**, ↑**UBA52**, ↑**ZFAND2A**	13	11	Drug metabolism, molecular transport, small molecule biochemistry
18	ABCD3, ACTC1, AFG3L1, ATP5B, ATP6V0B, ↑**ATP6V1G2**, ATPase, ↑**BAT1**, ↑**C20ORF7**, ↓**CHMP7**, ↑**CHMP2A**, CHMP4A, CHMP4B, CHMP4C, ↑**CSAD**, DDX1, DDX39, DNAJC14, ESCRT3, ESRRA, HLTF, MIRN324, MT-ATP8, MYH1, MYO9A, PPP2R4, RECQL5, RFC3, ↑**SARNP**, SKIV2L, ↓**STAMBP**, ↑**THOC2**, VPS24, VPS4B, ↓**ZC3H4**	12	10	Molecular transport, DNA replication, recombination, and repair, energy production
19	26s Proteasome, Actin, ATXN3, DNAJB2 (includes EG:3300), ↑**GM10117**, H2-LD, Histone h3, ↓**HNRNPK**, Hsp90, ID3, IKK (complex), Interferon-α, ↓**KEAP1**, Mapk, MAPK6, MOS, ↑**MYH14**, NEB, PACRG, PLA2G6, ↑**PPAN-P2RY11**, RAB8B, ↑**ROCK2**, RPS3, SNCAIP, ↑**TES**, ↑**TMCO6**, Tropomyosin, TRPM7, Ubiquitin, UBQLN1, USP2, ↑**USP3**, USP18	10	9	Cellular assembly and organization, cell cycle, cellular movement

Genes indicated in bold with either an up or down arrow were those found in data set. Genes in normal font are genes that IPA used to connect the genes together within networks. *Denotes genes found in >1 network.

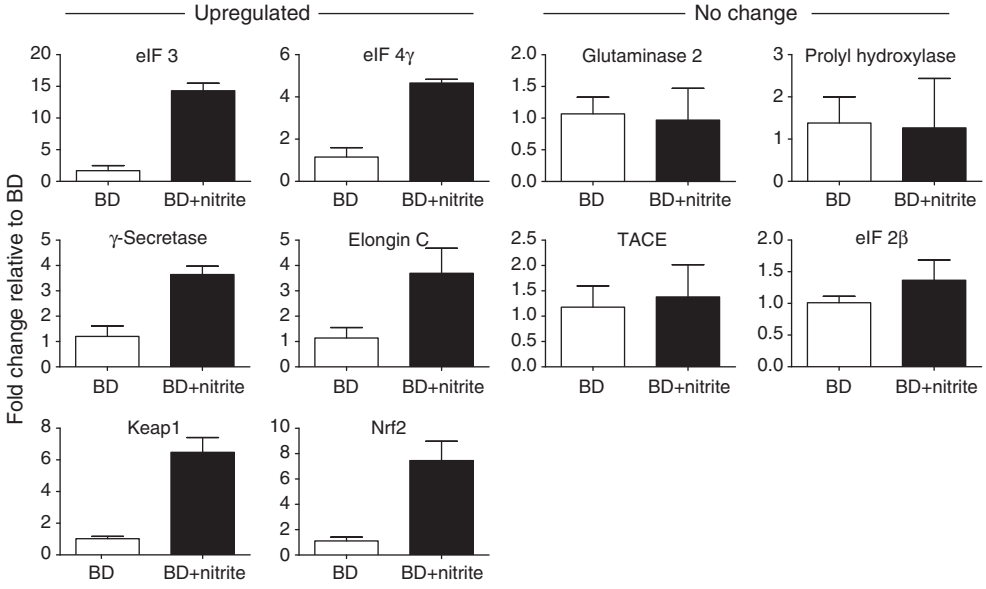

Figure 5 | Effects of nitrite on brain death (BD; 2 h)–induced changes in gene expression in the kidney. Real-time PCR analyses of indicated genes were performed. Data show fold change relative to BD alone and are mean ± s.e.m., $n = 3$. For all upregulated genes, $P < 0.05$ by *t*-test.

organs collected from living donors. This is important because the extent of this injury is inversely related to organ function and graft viability and provides a target for therapeutic intervention to both limit brain death–induced injury and prevent secondary transplantation–induced renal dysfunction. Underlying brain death–induced injury is increased ischemia and inflammation. Recent studies have shown that rather than an inert metabolite of aerobic NO metabolism, at low concentrations (nM/μM), nitrite can serve as a source for NO specifically under hypoxic and/or acidic conditions. This concept has been demonstrated by (1) decreased nitrite levels during ischemic stress, with the concomitant increase in NO and NO–protein adducts;[13,35–38] (2) stimulation of NO signaling in hypoxic, but not normoxic, tissues;[24,39] and (3) nitrite therapy protecting against I/R injury in diverse organs systems by NO formation.[11–13,21,40,41] This, together with decreased circulating nitrite levels during brain death (Figure 1), consistent

with ischemic stress, provided the rationale for testing nitrite therapy to attenuate brain death–induced renal injury and improve function after transplantation.

Decreased circulating nitrite levels could be due to endothelial dysfunction, as previously reported with brain death,[29] and/or increased consumption, which has also been reported in the setting of ischemic tissue injury.[13] In fact, data showing that nitrite levels in the circulation rise and then fall despite continual administration over 2 h of brain death (Figure 1d) suggest that increased nitrite consumption is occurring. Renal nitrite levels did not change with brain death, suggesting that with the relatively mild (only 2 h of brain death) model used herein ischemic stress in the circulation is more prevalent. Further studies evaluating nitrite therapy using longer BD times that also more severely compromise organ function are clearly required.

Most previous studies have shown nitrite protection against IR injury to be mediated by NO formation. In this model, nitrite targets NO formation only in the ischemic tissue, where it can stimulate multiple signaling responses that result in inhibition of IR injury, including attenuating inflammation and oxidative stress, inhibiting cell death, promoting blood flow, and regulating mitochondrial respiration.[20,40] Importantly, all of these potential effects could have a role in the observed protective effects of nitrite during brain death. On the basis of the fact that brain death causes decreased nitrite only in the intravascular compartment, we posit that the protective effects of nitrite are mediated primarily in the circulation and perhaps by improving renal blood flow or attenuating leukocyte activation, which could explain decreased CD11a and CD11b levels in the kidney. Coadministration of C-PTIO did not reverse nitrite-dependent protection, suggesting that NO-independent mechanisms are operating to limit kidney injury. In addition, a role for xanthine oxidoreductase was difficult to assess as allopurinol itself prevented brain death–induced injury more so than nitrite, suggesting that increased reactive oxygen species production has a role in brain death–induced organ dysfunction, a conclusion supported by increased lipid peroxidation in the kidney (Figure 4a).

Despite the lack of understanding of where nitrite is exerting protective effects, it is clear that nitrite therapy affects gene expression profile in the kidney after brain death. Previous studies have shown changes in the expression of 60–90 genes using microarray analysis in kidneys 6 h after brain death.[34] Interestingly, differential profiles of genes being upregulated or downregulated are observed in the kidney after brain death, and encompassed genes that affected metabolism/transport, immune/inflammatory cell activation, growth/fibrosis, and defense/repair processes. We used gene arrays to gain insights into potential mechanisms by which nitrite protected against brain death–dependent renal injury and specifically those that are not revealed by analysis of candidate markers of inflammation and oxidative stress. IPA of networks affected by nitrite compared with brain death alone showed that many processes were potentially affected (Table 1), including gene expression and modulation of humoral immune response, the latter being consistent with nitrite inhibiting inflammatory stress.

Within the top five canonical pathways that were most significantly affected by nitrite therapy, only a small percentage of total genes were in fact modulated by nitrite (4–10%), suggesting a specificity of responses. Real-time PCR analysis was performed on selected genes encompassing these pathways. Supplementary Table S1 online shows ~50% concordance between changes in gene expression mediated by nitrite determined by array and real-time PCR analysis. Importantly, however, at least one gene from each of the top five pathways identified as changing in response to nitrite therapy was validated by real-time PCR (Figure 5). Two of the top five pathways relate to expression of eukaryotic initiation factors with nitrite therapy upregulating eIF4γ. The exact consequences of such changes are difficult to predict, but are consistent with high diversity of genes affected by nitrite,[33] as indicated in Table 1. The second pathway was related to Notch signaling, an increase of which has recently been shown to facilitate tubular repair after acute renal injury.[42] Nitrite therapy increased the expression of γ-secretase, suggesting that increased Notch cleavage and subsequent stimulation gene transcription will occur. Potential effects of nitrite on HIF1α and Nrf2 (pathways 3 and 4, respectively) are clearly of interest with respect to known mechanisms of brain death–induced renal injury. Interestingly, nitrite therapy upregulated elongin-C; the predicted consequence is limited activation of HIF1α and may represent an affect of nitrite in countering brain death–induced ischemic stress. One potential mechanism is possible improvement in renal blood flow via reactions with hemoglobin.[43,44] Moreover, nitrite therapy upregulated the expression of Keap1 and Nrf2. Keap1 binds Nrf2 in the cytosol, preventing the latter from inducing a variety of stress response genes that protect cells from oxidative and inflammatory stimuli. Although relatively little is known on how expression levels of Keap1 and Nrf2 mRNA affect responses to oxidative/inflammatory stimuli, these data suggest that nitrite may affect oxidative and nitrosative signaling via these key transcription factors. Further studies evaluating how protein levels and transcriptional activity of the aforementioned mediators (identified from array and real-time PCR studies) change during brain death injury in the presence or absence of nitrite therapy are a necessary next step to further defining mechanisms of nitrite protection.

The current studies evaluated nitrite therapy during brain death itself and not after transplantation-dependent inflammatory injury. However, numerous studies have shown protective effects of nitrite during organ transplantation and/or IR injury, and in most of these instances nitrite is effective regardless of how it is administered (dietary, intraperitoneal, intraorgan injection, or intravenous).[11,13,14,28,45] With respect to the kidney, however, the route of administration of nitrite appears to have an important role. Intraperitoneal or intravenous administration did not protect against renal I/R injury,[46] whereas topical administration did.[25,30] The mechanistic

basis for this difference remains unclear. However, intravenous nitrite administration does protect the kidney from brain death–induced injury. How nitrite is administered during brain death is also important when assessing safety. A key element in brain death–induced kidney injury is hypotension, as demonstrated by experimental studies showing decreased injury when hypotension is avoided.[4] Indeed, clinically, the management of hypotension is by administration of fluid and/or vasoconstrictors. Nitrite is a vasodilator, which raises concerns about using this anion as a therapeutic. Initial studies testing bolus nitrite administration decreased MAP compared with brain death alone, resulting in mortality. However, administration of nitrite as for the duration of brain death did not result in additional hypotension and did not promote mortality. These data show that in the setting of brain death, nitrite can be administered safely and it protects against brain death–induced renal injury; however, the route of administration is critical.

The limitations of this study include the lack of precise mechanistic insights into how nitrite prevents brain death–induced renal injury. Moreover, we note that a relatively mild (2 h) brain death injury model was used (the severity of brain death injury increases with increased time of brain death[4]). Despite these limitations, the data presented herein support the feasibility of NO-based and specifically nitrite-based therapy for limiting brain death–induced renal injury.

In summary, we have shown that nitrite can be administered safely in a rat brain death model and was able to mitigate the brain death–induced I/R injury to renal grafts and improve post-transplant renal function. Nitrite administration during 2 h of brain death resulted in decreased markers of infiltrating inflammatory cells in the kidney, and, more importantly, resulted in kidneys whose function was significantly improved after transplantation compared with an allograft obtained from a BD-alone rat. These data suggest that nitrite prevents ischemic/inflammatory tissue injury during the BD phase, which results in a kidney that is more resistant to subsequent IR injury after transplantation. Whether nitrite-dependent protection occurs by affecting inflammation, tissue perfusion, or the kidney responses to ischemic and inflammatory stress is not clear. Gene array studies clearly suggest, however, that modulation of renal responses to stress is likely to have a role.

MATERIALS AND METHODS

Animals

Male Lewis rats aged 8–12 weeks and weighing 250–300 g were used. Animals were fed a normal NaCl diet (AIN-76A) (Dyets, Bethlehem, PA) as previously described[47] and allowed free access to water. All procedures were approved by the institutional animal care and use committee at the University of Alabama at Birmingham.

Brain dead model

Brain death was induced by balloon inflation of a Fogarty catheter introduced into the subdural space through an occipital burr hole as described in detail in Supplementary Material online. Briefly, after anesthesia, rats were intubated and mechanically ventilated with intermittent positive pressure for 2 h. Arterial blood pressure was monitored continuously. All animals received 0.9 ml of lactated Ringer solution intravenously for 30 min during the 2-h period. A 1-mm hole was drilled through the skull 0.3 cm lateral to the sagittal suture, and then a No. 3 Fogarty catheter was inserted through the burr hole. For raising the intracranial pressure gradually, the balloon was inflated with 40 µl/min saline until respiration ceased. The absence of reflexes, apnea, and maximally dilated and fixed pupils confirmed the condition. The balloon was kept inflated during the entire 2-h follow-up, after which left and right kidneys were harvested. Left kidney was used for transplantation and right kidney for mRNA or tissue collection for subsequent analyses. MAP was controlled and maintained between 60 and 80 mm Hg by addition of lactated ringers solution and Hespan as necessary. We note that with bolus administration of nitrite fluid administration was not able to increase MAP (see Figure 2).

Nitrite therapy

Sodium nitrite (Sigma, St Louis, MO) was dissolved in sterile phosphate-buffered saline and administered intravenously either as a bolus injection (0.1 mg/kg, in a 100 µl volume) or by injecting 133 µl every 10 min (over 2 h, the first dose starting 10 min after induction of brain death), resulting in 0.0083 mg/kg/injection or 0.1 mg/kg over 2 h. Saline was used as a control for nitrite. Nitrite administration was started 10 min after brain death induction. C-PTIO and allopurinol were administered at 1 mg/kg after induction of brain death.

Kidney transplant

Kidney transplantation surgery (Lewis to Lewis syngeneic transplantation) was performed according to the method previously described,[48] and is also described in detail in Supplementary Material online.

Creatinine and BUN assay

Serum creatinine and BUN levels were assayed using a VetACE biochemistry machine (Alfa Wasserman, West Caldwell, NJ).

Histology and immunofluorescence

Paraffin-embedded kidney sections were deparaffinized and stained with the indicated antibodies as described in Supplementary Material online.

Western blotting

Western blotting was performed as described in Supplementary Material online.

Nitrite measurements

Nitrite was measured in the blood and kidneys as previously described and in Supplementary Material online.[16]

RNA isolation for gene analysis

Detailed methods are provided in Supplementary Information online. Briefly, kidneys were cut into 3×3 cm segments and flash-frozen in liquid N_2. Total RNA was then isolated using a protocol based on the interaction of phenol and guanidine with cellular components. Gene expression analysis was performed using the Rat Ref-12 BeadChip and iScan system from Illumina (San Diego, CA). The Rat Ref-12 BeadChips contain sequences representing $\sim$22,000 curated and putative genes and expressed sequence tags. Quality standards for hybridization, labeling, staining, background signal,

and basal level of housekeeping gene expression for each chip were verified. After scanning the probe array, the resulting image was analyzed using the GenomeStudio software (Illumina). Samples were normalized using a quantile procedure, and differential gene expression for control and treatment groups was performed via a custom Illumina analysis designed to eliminate background noise from the analysis by subtracting out the signal from negative beads ($P<0.05$). Gene lists were not adjusted for multiple testing.

Ingenuity Pathway Analysis

The data set was analyzed by using the IPA software (version 8.7: Ingenuity Systems, www.ingenuity.com). The data set contained gene identifiers and corresponding expression values and was uploaded into the application. Each identifier was mapped to its corresponding object in the Knowledge Base of Ingenuity. Fold change for the target molecules was used to display the genes whose expression was upregulated or downregulated. These Network Eligible Molecules were overlaid onto a global molecular network developed from information contained in the Knowledge Base of Ingenuity. Networks of Network Eligible Molecules were then algorithmically generated based on their connectivity. The Functional Analysis identified the biological functions that were most significant to the data set. Right-tailed Fisher's exact test was used to calculate a *P*-value determining the probability that each biological function assigned to that data set is due to chance alone. Canonical pathway analysis identified the pathways from the IPA library of canonical pathways that were most significant to the data set. The significance of the association between the data set and the canonical pathway was measured in two ways. (1) A ratio of the number of molecules from the data set that map to the pathway divided by the total number of molecules that map to the canonical pathway is displayed. (2) Fisher's exact test was used to calculate a *P*-value determining the probability that the association between the genes in the data set and the canonical pathway is explained by chance alone. Finally, we note that gene array analysis of control vs. BD-alone, or control vs. nitrite-alone, groups revealed significant and distinct gene expression profile changes compared with BD vs. BD + nitrite (not shown) groups. Our focus here was on understanding the therapeutic potential of nitrite, and hence we limit the discussion to gene expression changes in BD + nitrite vs. BD-alone groups only.

Real time-PCR

Primer sequences and real-time PCR analyses were performed as described in Supplementary Data online.

Statistical analysis

Data are presented as mean ± s.e.m. The *t*-test was used for comparisons between two groups. For the comparisons that involved more than two groups, analysis of variance and the Tukey/Bonferroni post test were used for analysis, with statistical significance considered at $P<0.05$.

DISCLOSURE

RPP is listed as a coinventor on a patent for the use of sodium nitrite for the treatment of cardiovascular diseases. All the other authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Supplementary Information. Methods.
Table S1. Comparison of mRNA array data with real time-PCR data for selected genes.
Figure S1. Panel A shows a heat map indicating changes in gene expression in BD vs. BD + nitrite.
Figure S2. Panels A-E show consecutively the top 5 canonical signaling pathways (ELF2 (Panel A), Notch (Panel B), HIF1α (Panel C), NRF2/oxidative stress (Panel D), eIF4/p70S6K (Panel E)) identified by Ingenuity Pathway Analysis of gene expression changes that were modulated by nitrite therapy relative to BD alone.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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