Functional, morphologic, and molecular characterization of cold storage injury

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Objective: **Cold storage is used to preserve tissue for later transplantation. There is particular interest in prolonging cold storage time for transplantation purposes. To date, the mechanisms that contribute to vascular dysfunction in response to cold storage are poorly understood. The present study aims to characterize cold storage injury of blood vessels on functional and molecular levels.**

Methods: **To assess vessel function of mouse aorta, isometric force measurements were performed in a Mulvany myograph after cold storage at 4°C for various intervals. Morphologic changes were judged by histologic analysis of aortic cross-sections. To characterize cold storage-induced alterations on RNA levels, microarray analysis with subsequent polymerase chain reaction analysis was performed.**

Results: **Cold storage for 2 days revealed significant impairment of vessel function with respect to potassium-induced vessel tone development and acetylcholine-induced vessel relaxation. Detailed analysis of acetylcholine-mediated vascular response using specific pharmacologic blockers revealed that calcium-activated potassium channels seem to be impaired after 2 days of cold storage. At this point, no severe histologic changes (eg, elastic fiber disruption) were visible. RNA expression of 24 genes was significantly changed due to cold storage even after 2 hours. These include genes associated with vessel tone development (prostaglandin E_3 receptor), cardiovascular function (adiponectin), electron transport chain (uncoupling protein 1), or calcium signaling (protein kinase A regulatory subunit 2b).**

Conclusions: **Long-term cold storage impairs vascular function, especially with respect to potassium signaling by calcium-dependent potassium channels. Microarray analysis confirmed impairment of pathways that are involved in calcium signaling and vascular function. Furthermore, various genes were significantly altered even after 2 hours, significantly before functional impairment was observed. (J Vasc Surg 2012;56:189-98.)**

Clinical Relevance: **In bypass surgery, vessels are stored ex situ for <2 hours and for vessel banking for up to 48 hours during decontamination before cryopreservation. We investigated the onset of storage-induced alterations. The experiments reveal various genes were altered significantly, even within the first 2 hours of cold storage. This is of major importance, because of the time delay between regulation of messenger RNA and protein level and functional consequences. The alterations described here on the molecular level occur before any alterations on morphologic or functional levels are obvious. These molecular alterations, however, may affect later graft function.**

Cold storage at 4°C is routinely performed for donor tissues and organs for transplantation purposes during graft transport from the donor to the recipient. Decreasing the storage temperature can slow enzymatic activity of biologic processes by 1.5-fold to 2-fold per each 10-K decrease in temperature.[1] However, sufficient tissue preservation occurs only within a narrow interval. Hypothermic and ischemic injury[2] contribute to cold storage injury as well as other tissue-specific phenomena, including adaptation to lowered shear stress for blood vessels.[3]

In the regular course of bypass graft surgery, vein and artery grafts are stored at room temperature for <2 hours.[4] However, it could be reasonable to keep vessel segments of patients with a desperate graft situation for later reimplantation should early graft failure occur. Here, cold storage is advised. For later cryopreservation, grafts are usually stored for 2 days while undergoing decontamination by antibiotic treatment.[5] Furthermore, isolated blood vessel allografts are used for transplantation purposes, such as for infected artificial vascular grafts.[6] Other uses may include allogenic replacement due to vessel stenosis in association with transplantations. In addition, vascular grafts are used as conduits for connecting the transplanted organ to the circulation such as in kidney or liver transplantation.

Maintenance of vessel function and, in particular, endothelial cell function seems important because endothelial injury during storage may promote later vessel thrombosis and vessel wall inflammation[7,8] and induce graft failure. Disturbance in vascular tone regulation is associated with an increased risk for clinical events.[9]

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This study was funded by an intramural grant provided by the MeDDrive Programme of the Medical Faculty Carl Gustav Carus of Technical University Dresden, Germany.

Author conflict of interest: none.

Additional material for this article may be found online at www.jvascsurg.org.

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The editors and reviewers of this article have no relevant financial relationships to disclose per the JVS policy that requires reviewers to decline review of any manuscript for which they may have a conflict of interest.

0741-5214/$36.00

http://dx.doi.org/10.1016/j.jvs.2011.12.012

The aim of the present study was to identify cold storage-associated alterations at the functional, morphologic, and molecular level for future improvement of cold storage conditions. In a first step, the time of onset of vessel dysfunction for mouse aorta was determined, and pathways participating in vascular function were characterized. In addition to this functional characterization, microarray analysis was performed to identify genes that are regulated during cold storage. These targets may contribute to cold storage injury or may participate in physiologic processes to preserve the vessel. Mouse aorta was chosen as a model because this tissue is well characterized with respect to vessel function assays.

METHODS

Experiments involving animals were approved by the University Commission on Animal Experiments with respect to the animal welfare regulations of Germany, which conform to the National Institutes of Health guidelines. Written permission for the study was obtained from the local authorities (24-9168.24-1/2009-19).

Animals and storage groups. Male C57Bl/6 mice (Janvier, Le Genest Saint Isle, France) were euthanized by carbon dioxide exposure. The thoracic aorta (without the aortic arch) was harvested and directly placed in ice-cooled TiProtec storage solution (Dr. F. Köhler GmbH, Bensheim, Germany). Perivascular fat tissue was removed under a binocular microscope. Absence of fat cells was checked for each aorta at fourfold magnification, and special care was taken that no fat cells remained at side branches or the vessel rims. Up to four vessel segments of approximately 2 mm were cut and stored for a minimum of 2 hours up to a maximum of 7 days at 4°C. Special care was taken that all study groups contained proximal and distal segments.

After cold storage at 4°C, vessel segments were transferred to prewarmed (37°C) phosphate-buffered saline solution (containing 119 mmol/L NaCl; 4.7 mmol/L KCl; 1.17 mmol/L $MgSO_4$; 1.18 mmol/L KH_2PO_4; 25 mmol/L $NaHCO_3$; 5.5 mmol/L glucose; 0.027 mmol/L ethylenediaminetetraacetic acid sodium salt; and 2.5 mmol/L $CaCl_2$) and vessel tone was assessed by function testing using isometric force measurement.

Isometric force measurement. Vessel function was assessed according to the method of Mulvany and Halpern,[10] as described previously.[11,12] Briefly, aortic rings (712 ± 7 μm internal diameter) were studied in a myograph (Power Lab/400, DMT-610M; ADInstruments, GmbH, Spechbach, Germany) in which rings were stretched with a resting tension equivalent to that obtained by exposure to an intraluminal pressure of 20 mm Hg. Maximum constriction was induced by exposure of vessel rings to a potassium-enriched solution (123.7 mmol/L KCl). For methodic reasons, two potassium stimulations were performed and the second constriction was evaluated for receptor-independent response.

Receptor-mediated vessel tone was measured as the contractile response to 0.3 μmol/L phenylephrine. Vessel tone development is expressed as developed force relative to wall length.[10] Relaxation responses toward acetylcholine ($10^{-9.0}$ to $10^{-4.5}$ mol/L) and sodium nitroprusside ($10^{-10.0}$ to $10^{-4.0}$ mol/L) stimulation were tested after preconstriction was induced with phenylephrine to assess endothelium-dependent and endothelium-independent relaxations, respectively. These measurements were only performed for vessels with a sufficient preconstruction (>0.02 mN/mm). Also tested were responses to 11,12-epoxyeicosatrienoic acid (leukotriene response, $10^{-8.0}$ to $10^{-6.0}$ mol/L) and prostaglandin E_2 (prostaglandin response, $10^{-10.0}$ to $10^{-6.5}$ mol/L).

To evaluate the effects on different pathways, inhibitors were added 10 minutes before preconstriction with phenylephrine and dose-response curves with acetylcholine were performed. Inhibitors used for the following targets were endothelial nitric oxide synthases (eNOS): 100 μmol/L N^G-Methyl-L-arginine acetate salt (L-NMMA); neuronal NOS (nNOS)/inducible NOS (iNOS): 50 μmol/L 1-(2-Trifluoromethylphenyl)imidazole (TRIM); cyclooxygenase (COX): 1 μmol/L indomethacin; thromboxane A_2/prostaglandin H receptor: 1 μmol/L SQ29548; small and intermediate conductance calcium-gated potassium channels: combination of apamin (50 nmol/L) and charybdotoxin (50 nmol/L); potassium adenosine triphosphate (K_{ATP}) channels: 3 μmol/L glibenclamide.

Morphologic analysis of aorta cross-sections. To check for major morphologic alterations, aortic segments stored 2 hours or 2 days at 4°C in TiProtec solution and nonstored controls (n = 4 each) were fixed in formalin and embedded in paraffin. Cross-sections (4 μm) were stained according to elastica van Giessen and picro Sirius red standard protocols and examined under a light microscope to assess loss or disruption of elastic fibers and connective tissue, respectively. Smooth muscle cell organization was checked by immunohistochemistry using antimouse α-actin antibody (Abcam, Cambridge, UK).

RNA isolation, microarray, and real-time reverse transcription polymerase chain reaction. After the respective storage duration, vessels were placed in RNAlater solution (Ambion GmbH, Darmstadt, Germany) and snap-frozen in liquid nitrogen. Warm control vessels were dissected in RNAlater solution. RNA was isolated by the PeqGOLD TriFast kit (Peqlab, Erlangen, Germany) according to the manufacturer's standard protocol, with one exception: a double chloroform extraction was necessary to obtain sufficient RNA purity.

Alterations on the molecular level were evaluated by quantitative real-time reverse transcription polymerase chain reaction (RT-PCR; n = 6-8). For this purpose RNA was treated with DNaseI (Promega, Mannheim, Germany) to remove residual DNA and reverse transcribed with RevertAid HMinus First Strand cDNA Synthesis kit (Fermentas, St. Leon-Rot, Germany) using oligodT. Real-time RT-PCR was performed using the iCycler IQ Real Time PCR System (Bio-Rad, Hercules, Calif) and SYBR Premix ExTaq II (Lonza AG, Cologne, Germany). PCR conditions for all primer sets (Table I) were as

Table I. Primer for quantitative real-time reverse-transcription polymerase chain reaction analysis

Gene	*Sense primer*	*Antisense primer*
Acaa1	TTGCAAGTCAGGCCGTCTAC	GTGACAACCTGCCTTGCTCC
Adipoq	GAGATGCAGGTCTTCTTG	CGATACACATAAGCGGCTTC
AplnR	GCACCTGCATCAGTTATGTC	CTGATCACAGCAGAGCATGG
ApoCI	TGAGGCTCTTCATCGCTCTT	CCGGTATGCTCTCCAATGT
C1qa	GGCAATCCAGGCAATATCAG	AGATAACCACGTTGCCAAGC
Car3	ATGACAGGTCTATGCTGAGG	TGCTCAGAGCCGTGGTCATC
CD36	GATTAATGGCACAGACGCAG	GAACACAGCGTAGATAGACC
Ces3	TGCTGGTCTGGTTGCTACTC	GTCTCCAGTAGTTCATCCTC
CfD	ACTGCATGGATGGAGTGACG	CTGCACATCATACCATCGCT
Chchd10	ACAATCACGGTCTGAGCTCC	GAACGATCCTTCCTTACTCC
Cox7a1	CTTCCAGGCCGACAATGACC	GCAGTATAAGCAGTAGGCAG
Cox8b	GTCTCTGCCAAGCCAGCCAA	CATCCTGCTGGAACCATGA
Dbp	ACCGCGCAGGCTTGACAT	TTGGTTGAGGCTTCAGTTCC
Dgat2	GCCATGGAGCTGATCTG	CTCGGCCATGGAAGATGC
Dnm3os	GTGCAGGAAGACAGGCTCTC	GTCCTGAACAGGTAGTCTGA
Fabp4	GGTGAAGAGCATCATAACC	CCAGCTTGTCACCATCTCGT
Hp	GGTCCAGCCTATCTTGAACG	GTCCTCCTCCATGTCATGAA
Jun	ACGACCTTCTACGACGATGC	GCCAGGTTCAAGGTCATGCT
JunB	ACGGCCACCATCAGCTACCT	TACGGTCTGCGGTTCCTCTT
mAldn1a7	GCTGGCTACAATGGAATC	CAGCCTGCGAAGTACTGTAA
mElovl6	CCGAACTAGGTGACACGATA	ACCAGGAGTACAGGAGCACA
mGPR34	TAATGTGGCTGTTGCAGACC	TCACACCTAGTGTCCACTTG
mLyve1	ACAACTCATCCGACACCTG	GGATGAAGCCAAGTAGG
mNfkbiz	CGTGAATGCCAAGGCTTAC	CTGCGTCCAACTGTGTCACC
mOtop1	CCAACAGCGTCCTGAATGAA	CACTGTGGTGTGTGGTCATC
mPck1	GGTGTCATCCGCAAGCTGAA	CTGTGTCTCTCTGCTCTTGG
mPrkar2b	CCAAGGTGATGACGGTGACA	TAGTTACCGACGCATCTTCC
Mrap	GCATCAGCTGGCCTCACACT	CGACTGGCTCTCTGGCATCA
Orm1	GAGTACAGGCAGGCAATTCA	CCGAAGCTCTATTGTGTCGT
P2y12	GATTCAGCAGAACCAGGACC	GCCAAGCTGTTCGTGATGAG
Pde4b	GGTGATTGACATGGTGTTGG	GAGGAGAACACCGGAGCTTG
Polr2a	TCGAGCAGATCAGCAAGGTG	CAATGCCCAGTACCGTGAAG
Pon1	ACTTCTGCCTAGCATCAACG	ACATTGGACCACGACAGACC
PtgER3	GGTCGCCGCTATTGATAATG	GAATTGCACTCCTTCTCC
Retn	CAGTCTTGAGCTGCTCCTGT	GCTGCTGTCCAGTCTATCCT
Rnpc3	TTGGACCGATTACTGCACG	CCTCATCCTCATCTGGTAAG
Scd1	CCTTCCTCCTGAATACATCC	CTGCAGTGAACTTCATCAGC
Slc16a4	CTGCCAGTATTGGTTGACCT	CTGCTATTGGTGGTCCAGAA
Slc36a2	GCCACTGGTAGCAAGCTGGA	AGAGGCAGGACCACACCGAT
Tbp	CTCTGGAATTGTACCGCAGC	CGCAGTTGTCCGTGGCTCTC
Thrsp	CGGAACATGGAGCAGGTGGT	CTTGAGCATGGTGAAGTAGG
Timp4	ACTCGGCTCTAGTGATACG	ATCCTTGGCCTTCTCGAACC
Tmem45b	GTCAGCAGGAGCGCATTGAG	GGTGAGGTCCATCTGGAACA
Ucp1	TACCAAGCTGTGCGATGTCC	GGTACAATCCACTGTCTGTC

follows: 95°C for 2 minutes, followed by 40 amplification cycles, each consisting of 95°C for 20 seconds, 58°C for 45 seconds, 72°C for 20 seconds, and 76°C for 5 seconds, with a final extension step at 72°C for 2 minutes. Identity of PCR products was proved by melting point analysis and electrophoresis of the resulting PCR fragment. TATA binding factor (*Tbp*) and polymerase RNAII polypeptide A (*Polr2A*) served as housekeeping genes.

Data analysis and statistics. Vascular tone and vessel relaxation parameters were calculated as described previously.[11] Vascular tone is given as developed force relative to segment wall length. Maximum relaxation was expressed as the percentage of phenylephrine constriction. Here, a direct comparison was only possible for groups with similar preconstriction. The half-maximum effective dose (EC_{50}) was calculated from sigmoidal-fitted dose-response curves with Sigma Plot 10.0 software (Systat Software Inc, Chicago, Ill). Data are presented as mean ± the standard error of the mean.

Data were analyzed for normal distribution with Kolmogorov-Smirnov test. Homogeneity of variances was assessed according to Levene. Intergroup differences were tested using one-way analysis of variance. Differences were assessed with post hoc multiple comparisons. A value of $P \leq .05$ was taken to indicate a significant difference. Data analysis was performed using SPSS 11.5 software (SPSS Inc, Chicago, Ill).

For quantitative PCR analysis, relative expression was calculated by $^{2-\Delta\Delta Ct}$ methods using geometric average calculation to normalize for multiple housekeeping genes.[13] Data were standardized to a heart tissue standard.

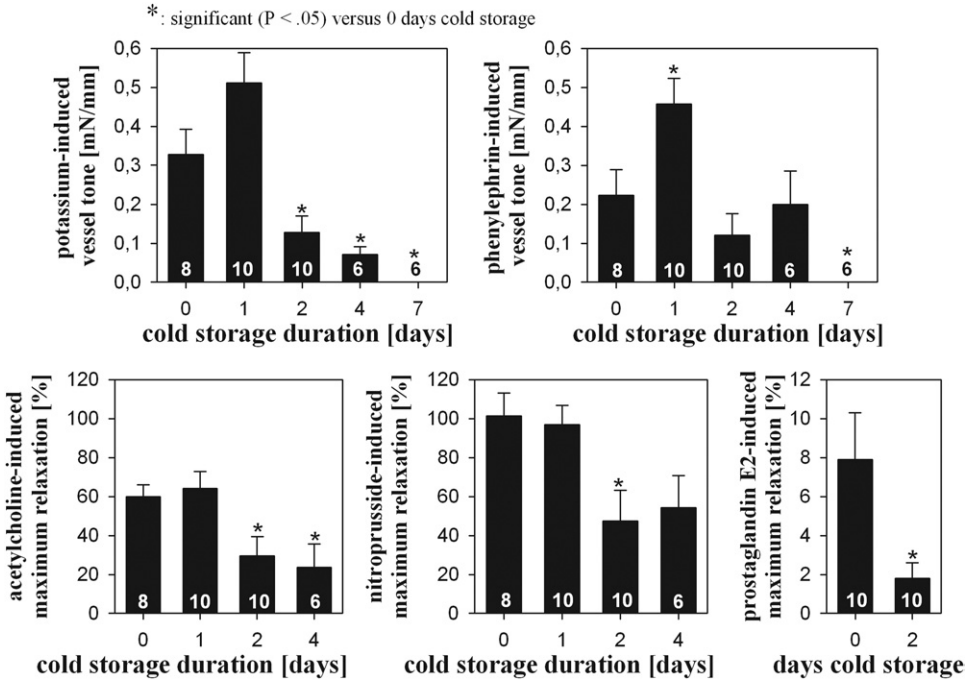

Fig 1. Vessel function after cold storage at 4°C in an optimized storage solution. Control vessels (0 days) were stored for 2 hours. Cold storage for 2 days significantly impairs vascular function with respect to potassium-induced constriction and acetylcholine as well as nitroprusside-induced relaxation compared with control conditions. The *error bars* show the standard error of the mean.

RESULTS

Vascular function of mouse aorta after cold storage. Cold storage at 4°C for ≥2 days significantly impaired function of mouse aorta. This was evident with respect to vessel tone development induced by a high potassium concentration as well as with respect to maximum vessel relaxation induced by acetylcholine and nitroprusside (Fig 1). Initially, a significant increase in vascular tone development was evident after 1 day of cold storage. Whether this may be attributed to recovery from the surgical trauma remains unclear at the present. After 7 days of cold storage, no preconstriction with phenylephrine was possible. Therefore, a subsequent relaxation analysis was not performed.

Endothelium-dependent vessel relaxation: Nitric oxide. The response toward acetylcholine is regarded an endothelium-dependent response because acetylcholine leads to a release of mediators from the endothelial cell layer, which in turn activate potassium channels that trigger smooth muscle relaxation.[14] In mouse aorta, vessel relaxation in response to acetylcholine is mostly mediated via nitric oxide release from eNOS, because only a small residual relaxation is observed in presence of L-NMMA (Fig 2, *A*). Presence of TRIM, an inhibitor of neuronal and inducible nitric oxide synthase (nNOS and iNOS) did not alter the maximum relaxation response of acetylcholine.

The maximum relaxation response was not significantly reduced in presence of L-NMMA, although a trend ($P < .20$) for reduction was obvious. The presence of L-NMMA influenced the relaxation sensitivity, calculated as the EC_{50} value. A significant shift of the dose-response curve to the right was observed, indicating an influence on relaxation sensitivity. This shift was significantly more pronounced in vessels stored for 2 days at 4°C (Fig 2, *B*).

Endothelium-dependent vessel relaxation: Arachidonic acid products. Arachidonic acid is metabolized by COX to a variety of vasoactive substances, including prostacyclin, prostaglandin E_2, or thromboxane A_2.[15] Prostaglandin E_2 only induced a small relaxation response in very low concentration (<3 nmol/L) in mouse aorta (Fig 1). This relaxation response was nearly completely abolished after 2 days of cold storage.

Indomethacin inhibition of COX activity does not alter maximum acetylcholine-induced relaxation (Fig 2, *A*). However, the dose-response curve in presence of indomethacin was significantly shifted to the right for both storage conditions (Fig 2, *B*), indicating reduced relaxation sensitivity and therefore a minor contribution of COX products to acetylcholine-induced relaxation.

Endothelium-dependent vessel relaxation: Endothelial cell hyperpolarization factor. Apart from nitric oxide and COX products, acetylcholine-induced relaxation may be mediated by a chemically unidentified stimulus

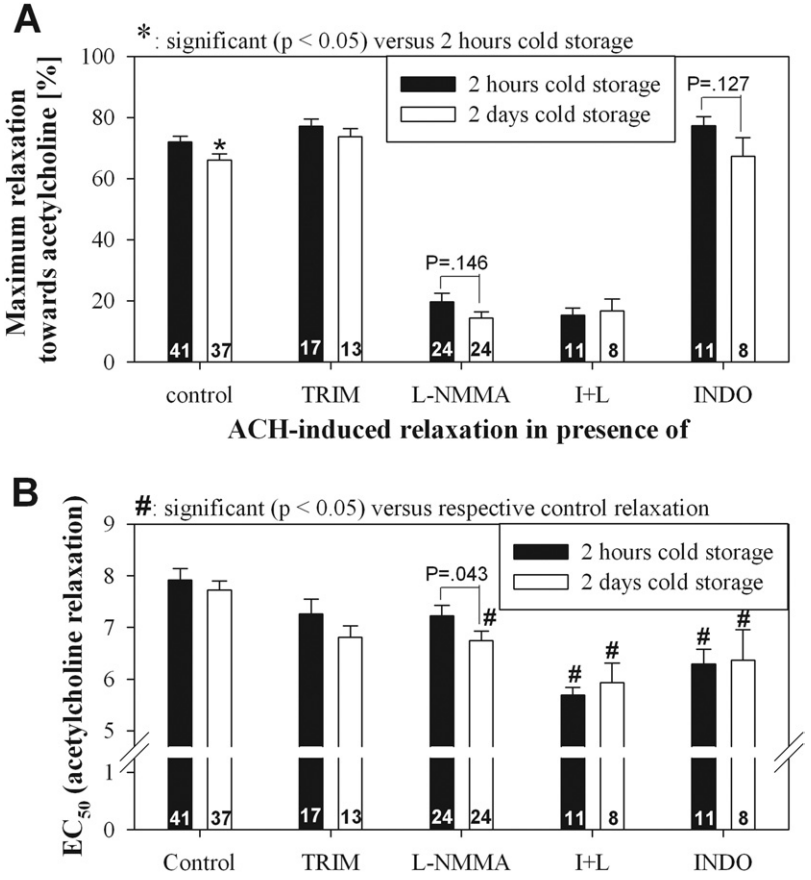

Fig 2. Contributions of different endothelium-derived mediators to acetylcholine (*ACH*)-dependent vessel relaxation: **(A)** maximum relaxation, **(B)** relaxation sensitivity (half-maximum effective dose [EC_{50}]). Relaxation in mouse aorta vessel is mostly mediated via nitric oxide synthase signaling, because only a small residual relaxation is observed in the presence of L-N^G-monomethyl arginine (*L-NMMA*). Inhibition of cyclooxygenase by indomethacin (*INDO*) does not alter maximum relaxation. In the combined presence of indomethacin and L-NMMA (*I+L*), no impairment of residual relaxation by cold storage was observed. The *error bars* show the standard error of the mean.

termed "endothelial cell hyperpolarization factor," which is defined to be the endothelium-dependent relaxation response persisting block of eNOS and COX.[16] In the combined presence of L-NMMA and indomethacin (I+L), no impairment of the minimal residual relaxation by cold storage was observed (Fig 2, *A*), and EC_{50}-values were similar ($P = .516$; Fig 2, *B*). In addition, the relaxation response toward 11,12-EET, a putative endothelial cell hyperpolarization factor mediator,[17] resulted only in a small contribution to relaxation (10.8% ± 2.0%), and this relaxation response was not significantly altered by prolonged cold storage (10.8% ± 1.7%).

Endothelium-dependent vessel relaxation: Potassium channels. Subsequent smooth muscle relaxation is mediated via ATP-gated potassium channels (K_{ATP}, blocked by glibenclamide) and calcium-activated potassium channels (K_{Ca}, blocked by apamin plus charybdotoxin). Impairment of acetylcholine-dependent vessel relaxation after 2 days of cold storage was observed in the presence of glibenclamide (Fig 3), but not in the combined presence of charybdotoxin and apamin. This indicates that the relaxation mechanisms that are impaired by 2 days of cold storage in mouse aorta mediate their relaxation via K_{Ca} channels.

Acetylcholine-dependent vessel contraction. Mouse aorta displays a biphasic response to acetylcholine (Fig 4, *left panel*), as others have described.[18] In low concentrations (<0.3 μmol/L), acetylcholine induced relaxation of preconstricted vessels; in higher concentrations (>1 to 10 μmol/L), acetylcholine caused slight vasoconstriction (Fig 4). This vasoconstriction is significantly ($P = .042$) reduced in aorta segments stored for 2 days (8% ± 2%) compared with segments stored for 2 hours (18% ± 5%). Acetylcholine-induced vasoconstriction is blocked by inhibitors of nNOS/iNOS (TRIM), thromboxane A_2/prostaglandin H-receptor (SQ29458), or COX (indomethacin).

Morphologic alterations by prolonged cold storage. Cross-sections were stained to assess elastic fibers and connective tissue to judge major morphologic damage, which may influence vessel tone development (Fig 5). Despite the

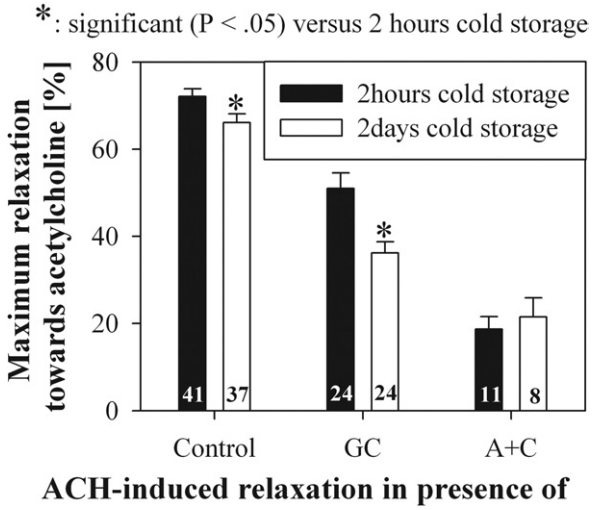

Fig 3. Contributions of different potassium channel types to acetylcholine (*ACH*)-dependent vessel relaxation: Impairment of acetylcholine-dependent vessel relaxation was observed in the presence of the adenosine triphosphate-dependent potassium channel inhibitor glibenclamide (*GC*) but not in the combined presence of charybdotoxin and apamin (*A+C*), inhibitors of large and intermediate calcium-sensitive potassium channels. The *error bars* show the standard error of the mean.

significantly impaired function (see above), neither loss nor disruption of connective tissue or elastic fibers was found after 2 days of cold storage.

PCR analysis. Microarray analysis (Appendix, online only) revealed that 36 annotated genes were significantly altered by more than threefold. These and additionally phosphodiesterase 4b (*Pde4b*), purinergic receptor (*P2y12*), tissue inhibitor of metalloproteinases 4 (*Timp4*), D site albumin promoter binding protein (*Dbp*), complement factor 1 (*C1qa*), and *JunB* oncogene were analyzed by quantitative real-time RT-PCR for validation of microarray results. For RNA analysis, it was methodically possible to introduce a warm control group (no cold injury or contact with the storage solution) that served as a nondamaged control. Quantitative RT-PCR showed that 24 genes were significantly changed due to cold storage. Results are summarized alphabetically in Table II. Most genes, with the exception of stearoyl-coenzyme A desaturase 1 (*Scd1*), melanocortin two-receptor accessory protein (*Mrap*), lymphatic vessel endothelial hyaluronan receptor 1 (*Lyve1*), and *JunB* oncogene were altered significantly even within the first 2 hours of cold storage.

DISCUSSION

Mouse models are frequently used for research purposes because they are cheap, well characterized, and offer unique possibilities due to the availability of a variety of genetically modified strains.[19] Murine models of vessel transplantation studies usually use the thoracic aorta.[20] The present study characterizes cold storage injury of mouse aorta on functional and molecular levels.

Application of sodium nitroprusside, a compound directly releasing nitric oxide, results in a complete relaxation of phenylephrine preconstriction, and this response was significantly reduced after 2 days of cold storage. This indicates failure of nitric oxide signaling downstream of nitric oxide release from endothelial cells. Approximately 72% of a preconstriction with phenylephrine can be relaxed using acetylcholine. The nitric oxide-mediated part of the acetylcholine response can be estimated by subtraction of the residual relaxation in presence of L-NMMA from control relaxation. This was in the same range for vessels stored 2 hours (54% ± 4%) and for segments stored for 2 days (54% ± 3%). Therefore, impaired eNOS signaling is unlikely to be the reason for impaired acetylcholine relaxation.

Combined application of L-NMMA and indomethacin reduced the mean maximum relaxation by 4% after 2 hours of cold storage and significantly lowered the relaxation sensitivity compared with the application of L-NMMA

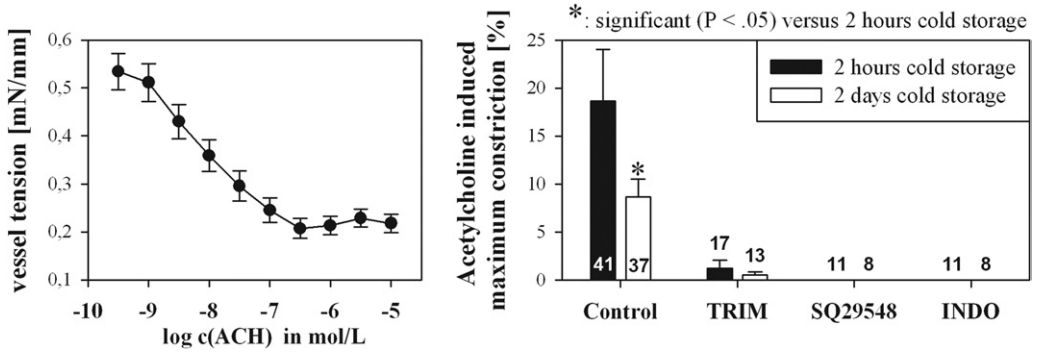

Fig 4. Acetylcholine-dependent vessel tone modulation. **Left panel,** Mouse aorta displays two distinct responses to acetylcholine (*ACH*). In low concentration (<10 to 6.5), ACH is able to relax a preconstricted vessel; in higher concentrations (>10 to 6), relaxation ceases and turns toward vasoconstriction. **Right panel,** ACH-induced maximum vasoconstriction (expressed as percentage of phenylephrine preconstriction) is impaired by 2 days of cold storage. This response is blocked by inhibitors of neuronal and inducible nitric oxide synthase (*TRIM*), thromboxane A2/prostaglandin H receptor (*SQ29548*), or cyclooxygenase (*INDO*). The *error bars* show the standard error of the mean.

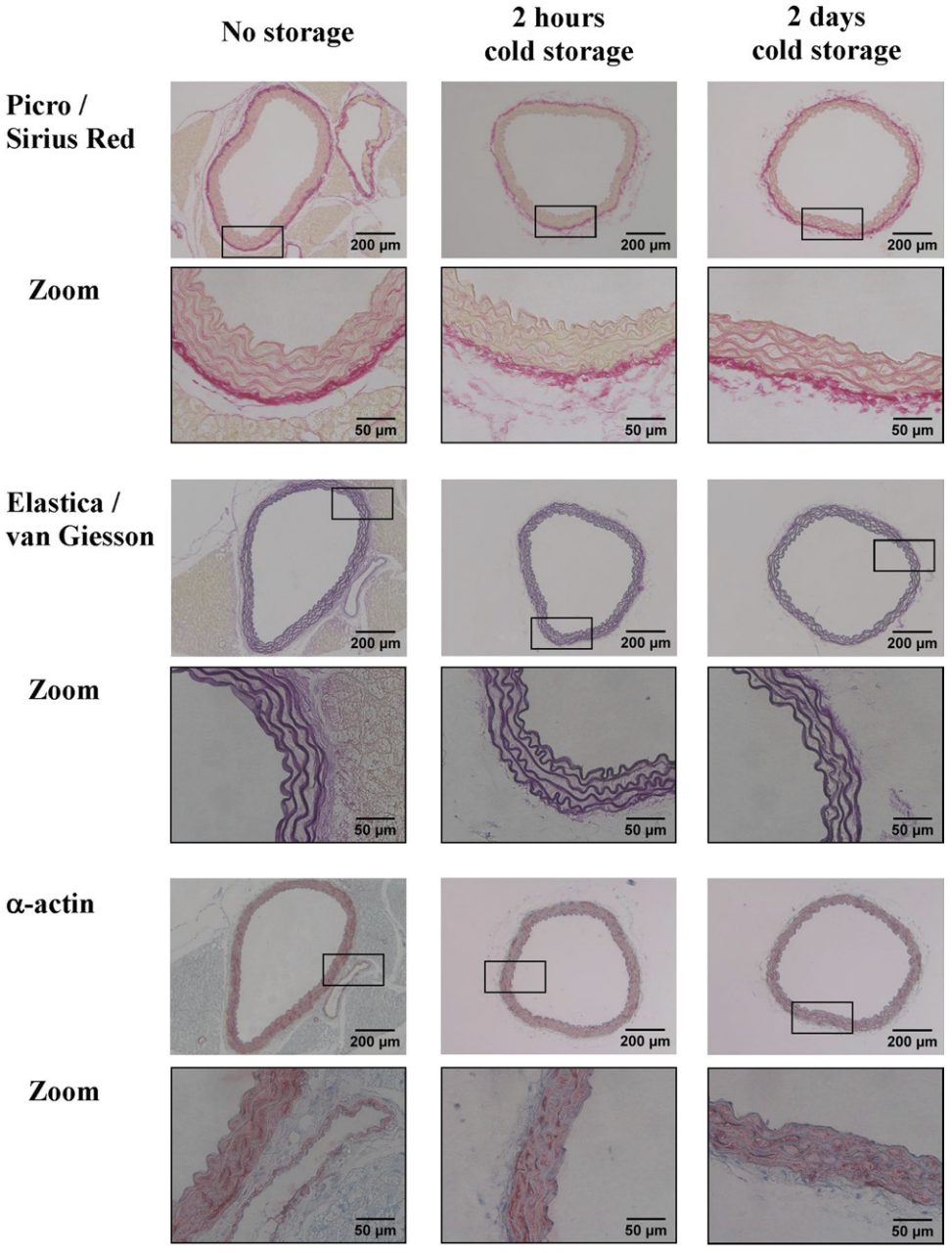

Fig 5. Staining with **(top)** picro-Sirius red and **(middle)** elastica van Giessen, and **(bottom)** α-actin immunohistochemistry of aorta cross-sections shows no loss or disruption of connective tissue (*red* in the *top panel*) and that elastic fiber (*black-lilac* in the *middle panel*) was induced by prolonged cold storage. Smooth muscle cell organization was not disturbed. Original magnification was 100× for overview pictures and 400× for zoom views.

alone. This was not the case after 2 days of cold storage, suggesting an involvement of COX products in cold storage injury. This finding supports the hypothesis that the minimal relaxation response toward prostaglandin E_2 and the acetylcholine-mediated constriction are reduced after 2 days of cold storage. Evidence from the current study and work from others shows that this vasoconstriction is mediated by superoxide radicals or COX products, or both, that are released from the endothelium of mouse aorta.[18]

This leaves the question of which downstream effects of nitric oxide signaling (sodium nitroprusside response) are impaired. Downstream relaxation mechanisms may include activation of ATP-dependent or calcium-gated potassium channels, or both.[21] In the present study, approximately one-third of the acetylcholine response was blocked in presence of glibenclamide, an inhibitor preferentially affecting K_{ATP} channels, and two-thirds were blocked by the combined presence of apamin and charybdotoxin. The

Table II. Results for polymerase chain reaction validation[a]

Gene symbol	*Gene title*	*Function*	*Control*[b]	*2 Hours*[c]	*2 Days*[d]
Adipoq	Adiponectin	Adipokine	76 ± 22	21 ± 6[e]	13 ± 1[e]
ApocI	Apolipoprotein C-I	Uptake and metabolism of VLDL and HDL particles	12 ± 5	6.1 ± 1.0[e]	6.2 ± 1.1[e]
Car3	Carbonic anhydrase 3	EC 4.2.1.1: percarbonic anhydrase	77 ± 24	22 ± 5[e]	16 ± 4[e]
CD36	CD36 antigen	Transmembrane receptor (various ligands)	0.09 ± 0.03	0.04 ± 0.00[e]	0.05 ± 0.01
Cfd	Complement factor D	EC 3.4.21.46: serine protease	100 ± 3	27 ± 8[e]	17 ± 5[e]
Cox8b	Cytochrome-*c* oxidase subunit VIIIb	EC 1.9.3.1: involved in electron transport chain	0.07 ± 0.02	0.02 ± 0.01[e]	0.02 ± 0.01[e]
Dbp	D site albumin promoter binding protein	Transcription factor	0.22 ± 0.04	4.9 ± 2.6[e]	3.4 ± 2.2
Dgat2	Diacylglycerol *O*-acyltransferase 2	EC 2.3.1.20: catalyses the final step of triacylglycerol synthesis	0.44 ± 0.17	0.07 ± 0.02[e]	0.07 ± 0.00[e]
Elovl6	ELOVL family member 6	Elongation of long-chain fatty acids	19 ± 5	8.0 ± 1.3[e]	6.3 ± 0.8[e]
Fabp4	Fatty acid binding protein 4	Regulates intracellular trafficking of long-chain fatty acids	0.65 ± 0.22	0.22 ± 0.04[e]	0.21 ± 0.05[e]
Jun	Jun oncogene	Regulator of transcription	0.95 ± 0.20	0.92 ± 0.20	2.1 ± 0.8[b]
Lyve1	Lymphatic vessel endothelial hyaluronan receptor 1	Hyaluronan uptake	1.07 ± 0.15	1.21 ± 0.10	0.94 ± 0.06[b]
Mrap	Melanocortin 2 receptor accessory protein	Trafficking and function of melanocortin receptor 2	1.01 ± 0.40	0.43 ± 0.11	0.22 ± 0.03[e]
Otop1	Otopetrin 1	Mineralization of otoconia	0.04 ± 0.01	0.01 ± 0.00[e]	0.00 ± 0.00[e]
Pck1	Phosphoenolpyruvate carboxykinase 1, cytosolic	EC 4.1.1.32: phosphoenolpyruvate carboxykinase (GTP)	69 ± 31	26 ± 4[e]	15 ± 2[e,f]
Pon1	Paraoxonase-1	EC 3.1.1.17: gluconolactonase	1.02 ± 0.38	0.39 ± 0.12	0.30 ± 0.07[e]
Prkar2b	Protein kinase, cAMP dependent regulatory, type Iβ	Fibroblast growth regulation	21 ± 10	6.1 ± 1.6[e]	3.7 ± 0.6[e]
Ptger3	Prostaglandin E receptor 3	Mediation of vasoconstriction	38 ± 10	19 ± 2[e]	20 ± 5[e]
Retn	Resistin	Adipokine	93 ± 28	26 ± 5[e]	20 ± 5[e]
Scd1	Stearoyl-coenzyme A desaturase 1	EC 1.14.19.1: energy metabolism	53 ± 19	22 ± 10	7.8 ± 1.0[e]
Slc36a2	Solute carrier family 36, member 2	Proton/amino acid symporter	2.3 ± 1.5	0.30 ± 0.07[e]	0.26 ± 0.07[e]
Thrsp	Thyroid hormone responsive SPOT14 homolog	Expressed upon fatty acid stimulation	2.10 ± 0.89	0.43 ± 0.12[e]	0.26 ± 0.07[e]
Tmem45b	Transmembrane protein 45b	Unknown	7.2 ± 2.6	1.7 ± 0.6[e]	0.81 ± 0.35[e]
Ucp1	Uncoupling protein 1	Mitochondrial proton carrier; involved in electron transport chain	3419 ± 1505	309 ± 124[e]	167 ± 78[e]

cAMP, Cyclic adenosine monophosphate; *HDL*, high-density lipoprotein; *VLDL*, very low-density lipoprotein.
[a]Relative expression was normalized to housekeeping genes *Tbp* and *Polr2A* and is given in relation to a heart tissue standard.
[b]Control: no cooling, no contact with storage solution.
[c]2 hours of storage at 4°C.
[d]2 days of storage at 4°C.
[e]Significantly altered compared with nonstored controls.
[f]Significant difference between 2 hours and 2 days of cold storage.

acetylcholine-induced relaxation persisting with the combined presence of apamin and charybdotoxin is therefore mediated through K_{ATP}-channels, and this response is not impaired by cold storage. Under block of K_{ATP}-channels, the residual relaxation was significantly lower after 2 days of cold storage. Relaxation mechanisms that are active in presence of glibenclamide may include small, large, and intermediate conductance Ca^{2+}-activated potassium channels as well as voltage-gated potassium channels.[22] Other relaxation mechanisms (eg, leukotriene-stimulated relaxation) are not impaired, however. We note that all functional changes were found in the absence of major morphologic alterations.

To characterize cold storage injury in mouse aorta on a molecular level, we performed microarray analysis and validated the results by real-time RT-PCR. Various genes were altered significantly, even within the first 2 hours of cold storage. This is of major importance because of the time delay between alterations on messenger RNA and the functional level. This means that regulation on the molecular level may occur before any alterations on the morphologic or functional level develop. This may be of utmost importance for peripheral or coronary artery bypass grafting, where vessel segments are stored for intervals of <2 hours. After 2 hours of cold storage, vascular function is not reduced compared with warm controls (acetylcholine relaxation warm control, 61%; cold storage, 60%).

RNA expression of various genes was significantly altered within this narrow time frame, however, and some of these targets likely have implications for later graft function. For example, protein kinase A regulatory subunit-β (*Prkar2b*) can regulate fibroblast growth and apoptosis, depending on the presence of thyroid-stimulating hormone.[23] Furthermore, Prkar2b is involved in suppression of balloon injury-induced neointimal hyperplasia.[24] Another example is prostaglandin E_3 receptor, which is known to mediate and potentate vasoconstriction in rat vessels.[25] Fatty acid binding protein 4 (*Fabp4*) regulates intracellular trafficking of long-chain fatty acids, and significantly higher expression has been described in the aortic tissue of patients with metabolic syndrome and severe coronary artery occlusion than in patients with a normal body mass index and aortic stenosis.[26]

Expression in the vascular system of other targets has not been described before, but their function in other tissues may link them to cold storage injury of mouse aorta. Uncoupling protein 1 (*Ucp1*) is a mitochondrial proton carrier that mediates brown adipose tissue hypoxia in response to hypothermia.[27] Carbonic anhydrase 3 (*Car3*) protects against radical injury that is induced by hydrogen peroxide (H_2O_2).[28] These Fenton-type reactions of H_2O_2 are crucially involved in cold storage injury in the absence of respective protective substances.[29] Some have speculated that Car3 is actually not a carbonic anhydrase ($H_2O + CO_2 \rightarrow H_2CO_3$) but a percarbonic anhydrase ($H_2O_2 + CO_2 \rightarrow H_2CO_4$) and therefore directly degrades H_2O_2.[30]

Adiponectin (*Adipoq*) and resistin (*Retn*) are adipokines that are associated with the outcome of peripheral revascularization.[31] Adipsin (complement factor D [*CfD*]) is a serine protease involved in the alternative pathway of complement immune response.[32] Diacylglycerol *O*-acyltransferase 2 (*Dgat2*) catalyses the final step of triacylglycerol synthesis and therefore crucially regulates triglyceride content in liver[33] and very low-density lipid proteins (VLDL) triglyceride content.[34] This may be of importance for hypertriglyceremia-induced atherosclerosis. Apolipoprotein C1 (*ApoCI*) is a potent inhibitor of hepatic uptake of VLDL particles and inhibits triglyceride hydrolysis.[35] Furthermore, ApoC1 regulated HDL lipid composition and elevated LSP-induced inflammatory response and atherosclerotic lesion formation at the aortic root of ApoE-deficient mice.[36]

Which of these genes determines graft quality and patency remains to be established. For many of these molecular targets (eg, secreted factors such as adiponectin, resistin, adipsin, and ApoC1), a direct functional implication of local expression changes for the vascular system has not been described. To date, only systemic functions have been documented, and production of these compounds by the graft tissue after altered expression may interact with that provided from other sites of production. For some of the targets (eg, *Tmem45b*), no function at all has been described to date.

Taken together, we demonstrate that cold storage of mouse aorta for 2 days results in a decline of vasorelaxation and stimulus-induced vasoconstrictor function. This dysfunction includes endothelial-dependent and smooth muscle-dependent pathways and is paralleled by alteration of various genes. Strikingly, many of the alterations observed on the molecular level occur within the first 2 hours of storage and may influence later graft function. The functional changes appear ≤2 days of cold storage yet without significant alterations of vessel structure.

CONCLUSIONS

Further studies are needed to evaluate the target–function relationship for each altered gene and elucidate the role of these targets for vascular function and graft patency after implantation. In addition, the exact onset of the observed alterations on molecular level needs to be analyzed in more detail in graft-relevant human vessel models. Furthermore, whether the disturbance in one of the molecular structures affects cold storage sensitivity remains to be investigated. This would be of particular interest for comorbidities that are associated with impaired vascular function.

The authors thank Birgit Zatschler and Peggy Barthel for technical assistance.

AUTHOR CONTRIBUTIONS

Conception and design: AE, AD
Analysis and interpretation: AE, DP, AA
Data collection: AE, DP
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Statistical analysis: AE
Obtained funding: AE, RS, AD
Overall responsibility: AD

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Submitted Sep 28, 2011; accepted Dec 5, 2011.

Additional material for this article may be found online at www.jvascsurg.org.

APPENDIX (online only)

Methods. Fat-free mouse aortas stored for 2 hours (n = 3) or 2 days (n = 3) at 4°C were studied. After the respective storage duration, vessels were placed in RNAlater solution (Ambion). RNA was isolated by PeqGOLD TriFast kit (Peqlab) according to the manufacturer's standard protocol with one exception: a double chloroform extraction was necessary to obtain sufficient RNA purity. Affimetrix whole mouse genome microarray analysis was performed at the microarray core facility of Max Planck Institute for Cell Biology and Molecular Genetics, Dresden, according to the manufacturer's instructions.

Data evaluation of the microarray analysis was performed according to manufacturer's suggestions using GeneSpring × 6 11.01 software (Agilent Technologies, Santa Clara, Calif). Data were compared as normalized quartiles. The 20% lowest expressed genes and mismatch genes were excluded from analysis; only genes with at least three valid measurements of the six analyzed samples measured were included. A value of $P < .05$ and at least a twofold change was considered significant.

Results. Microarray analysis was performed for three samples each of mouse aorta stored 2 hours or 2 days at 4°C in an optimized storage solution and revealed 118 significantly and more than twofold altered genes, of which 82 were annotated. These genes included 14 upregulated genes, and 68 of the significantly altered annotated genes were downregulated (Table III, online only). Gene ontology metabolic pathway analysis was performed. No clear picture on one specifically targeted pathway was observed, but a broad variety of genes that are known to be involved in many different metabolic functions were seen. Some of these pathways may be involved in the regulation of vascular function; for example, smooth muscle contraction (*Pde4b, Rgs18, Prkar2b, Jun*), prostaglandin synthesis and metabolism (*Hpgd, Endrb, Ptger3*), calcium regulation (*Prkar2b, Adr3b, Rgs18*), or electron transport chain (*Ucp1, Cox8b, Cox7a1, Atp5ca*).

Table III (online only). Microarray results

Gene symbol	*Gene title*	*Fold change*	P
Upregulated genes			
Jun	Jun oncogene	3.3	<.001
Nfkbiz	INAP messenger RNA for IL-1 inducible nuclear ankyrin-repeat protein	3.0	<.001
JunB	JunB oncogene	2.9	.001
Nr1d1	Nuclear receptor subfamily 1, group D, member 1	2.5	.013
Dbp	D site albumin promoter binding protein	2.5	.042
Hist1h1e	Histone cluster 1, H1e	2.4	.005
BtgI	B-cell translocation gene 1, antiproliferative	2.2	<.001
Trio	Triple functional domain (PTPRF interacting)	2.2	.006
Pde4b	Phosphodiesterase 4B, cAMP specific	2.2	.004
Gadd45g	Growth arrest and DNA-damage-inducible 45 gamma	2.1	.001
Timp4	Tissue inhibitor of metalloproteinase 4	2.1	.010
Picalm	Phosphatidylinositol binding clathrin assembly protein	2.1	.005
Fam107a	Family with sequence similarity 107, member A	2.1	.050
Fosb	FBJ osteosarcoma oncogene B	2.0	.047
Gene symbol	*Gene title*	*Absolute fold change*	P
Downregulated genes			
Ucp1	Uncoupling protein 1 (mitochondrial, proton carrier)	9.1	.001
Ces3	Carboxylesterase 3	7.9	.002
Adipoq	Adiponectin, C1Q and collagen domain containing	7.8	.009
Cox8b	Cytochrome-*c* oxidase, subunit VIIIb	7.1	.002
Car3	Carbonic anhydrase 3	6.9	.008
Thrsp	Thyroid hormone responsive SPOT14 homolog (Rattus)	6.9	.001
Dnm3os	Dynamin 3, opposite strand	6.7	<.001
Cfd	Complement factor D (adipsin)	6.5	.014
Tmem45b	Transmembrane protein 45b	5.2	.004
CD36	CD36 antigen	5.1	.004
Hp	Haptoglobin	4.9	.016
Cox7a1	Cytochrome-*c* oxidase, subunit VIIa 1	4.9	.001
Fabp4	Fatty acid binding protein 4, adipocyte	4.7	.005
Aldh1a7	Aldehyde dehydrogenase family 1, subfamily A7	4.5	.005
Orm1	Orosomucoid 1	4.2	.001
Slc16a4	Solute carrier family 16 (monocarboxylic acid transporters), member 4	4.0	<.001
Prkar2b	Protein kinase. cAMP dependent regulatory, type II beta	4.0	.005
Otop1	Otopetrin 1	3.8	.012
ApocI	Apolipoprotein C-I	3.8	.008
Scd1	Stearoyl-coenzyme A desaturase 1	3.6	.012
Rnpc3	RNA-binding region (RNP1, RRM) containing 3	3.6	.001
Slc36a2	Solute carrier family 36 (proton/amino acid symporter), member 2	3.5	.007
Ptger3	Prostaglandin E_3 (subtype EP3)	3.4	.001
Pck1	Phosphoenolpyruvate carboxykinase 1, cytosolic	3.4	.030
Retn	Resistin	3.3	.018
Aplnr	Apelin receptor	3.3	.007
Mrap	Melanocortin 2 receptor accessory protein	3.2	.003
Dgat2	Diacylglycerol *O*-acyltransferase 2	3.1	.003
Pon1	Paraoxonase-1	3.1	.031
Gpr34	G protein-coupled receptor 34	3.1	.008
Chchd10	Coiled-coil-helix-coiled-coil-helix domain containing 10	3.0	<.001
Acaa1b	Acetyl-CoA acyltransferase 1B	3.0	.008
Lyve1	Lymphatic vessel endothelial hyaluronan receptor 1	3.0	.022
Elovl6	ELOVL family member 6 (elongation of long chain fatty acids [yeast])	3.0	<.001
Gas5	Growth arrest specific 5	2.9	<.001
Ednrb	Endothelin receptor type B	2.9	.032
Fubp1	Far downstream element (FUSE) binding protein 1	2.9	.002
Fasn	Fatty acid synthase	2.8	.001
Gpd1	Glycerol-3-phosphate dehydrogenase 1 (soluble)	2.8	.002
CD163	CD163 antigen	2.7	.048
C1qa	Complement component 1, q subcomponent. Alpha polypeptide	2.6	.047

Table III (online only). Continued

Gene symbol	*Gene title*	*Fold change*	P
Cidec	Cell death-inducing DFFA-like effector c	2.6	.047
Pcx	Pyruvate carboxylase	2.5	.007
Acss2	Acyl-CoA synthetase short-chain family member 2	2.5	.001
Cyp2f2	Cytochrome P450, family 2, subfamily f. polypeptide 2	2.4	.035
Rbm3	RNA binding motif protein 3	2.4	.013
Adrb3	Adrenergic receptor, beta 3	2.4	.013
Clec4a3	C-type lectin domain family 4, member a3	2.4	.004
St8sia4	ST8 alpha-N-acetyl-neuraminide alpha-2.8-sialyltransferase 4	2.4	.005
Ccr5	Chemokine (C–C motif) receptor 5	2.4	.007
Hpgd	Hydroxyprostaglandin dehydrogenase 15 (NAD)	2.4	.027
Rbmx	RNA binding motif protein, X chromosome	2.4	.002
Chd2	Chromodomain helicase DNA binding protein 2	2.3	<.001
Ttc14	Tetratricopeptide repeat domain 14	2.3	<.001
Agpat2	1-Acylglycerol-3-phosphate *O*-acyltransferase 2	2.3	.001
Fcgr2b	Fc receptor, immunoglobulin G, low affinity IIb	2.3	.033
Prei4	Preimplantation protein 4	2.3	<.001
Atp5c1	ATP synthase, H+ transporting; mitochondrial F1 complex, gamma polypeptide 1	2.3	<.001
Xpa	Xeroderma pigmentosum, complementation group A	2.3	<.001
Slc16a1	Solute carrier family 16 (monocarboxylic acid transporters), member 1	2.3	.002
Emr1	EGF-like module containing. Mucin-like, hormone receptor-like sequence 1	2.3	.015
Rprd1a	Regulation of nuclear pre-mRNA domain containing 1A	2.3	.001
Cd1d1	CD1d1 antigen	2.3	.010
Dleu2	Deleted in lymphocytic leukemia, 2	2.2	.002
C3ar1	Complement component 3a receptor 1	2.2	.018
Ms4a6d	Membrane-spanning 4-domains, subfamily A, member 6D	2.2	.040
Mgl2	Macrophage galactose N-acetyl-galactosamine specific lectin 2	2.2	.039
Pstk	Phospho seryl-tRNA kinase	2.2	.001
Lipe	Lipase, hormone sensitive	2.2	.001
Ccl9	Chemokine (C-C motif) ligand 9	2.2	.043
Malat1	Metastasis associated lung adenocarcinoma transcript 1 (noncoding RNA)	2.1	.005
RGS18	Regulator of G protein signaling 18	2.1	.015
Pus3	Pseudouridine synthase 3	2.1	.005
Tardbp	TAR DNA binding protein	2.1	.001
Acly	ATP citrate lyase	2.1	.001
Cidea	Cell death-inducing DNA fragmentation factor, alpha subunit-like effector A	2.1	.018
Zfp207	Zinc finger protein 207	2.1	.006
Gpihbp1	GPI-anchored HDL-binding protein 1	2.1	.002
P2ry12	Purinergic receptor P2Y, G-protein coupled 12	2.0	.007
Zfp58	Zinc finger protein 58	2.0	.025
CD53	CD53 antigen	2.0	.011
Cebpa	C/EBP, alpha	2.0	.003
Mirhg1	microRNA host gene 1	2.0	.009
Rbbp6	Retinoblastoma binding protein 6	2.0	.010
Lyz2	Lysozyme 2	2.0	.045
F13a1	Coagulation factor XIII, A1 subunit	2.0	.041

ATP, Adenosine triphosphate; *cAMP,* cyclic adenosine monophosphate; *C/EPB,* cytidine-cytidine-adenosine-adenosine-thymidine enhancer-binding proteins; *CoA,* coenzyme A; *GPI,* glycosylphosphatidylinositol; *EGF,* endothelial growth factor; *HDL,* high-density lipoprotein; *IL,* interleukin; *INAP,* inducible nuclear ankyrin-repeat protein.