Identification of apolipoprotein D as a cardioprotective gene using a mouse model of lethal atherosclerotic coronary artery disease

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Mice with homozygous null mutations in the HDL receptor (scavenger receptor class B, type I, or SR-BI) and apolipoprotein E (apoE) genes [SR-BI/apoE double KO (SR-BI$^{-/-}$/apoE$^{-/-}$ or dKO) mice] spontaneously develop occlusive, atherosclerotic coronary artery disease (CAD) and die prematurely (50% mortality at 42 d of age). Using microarray mRNA expression profiling, we identified genes whose expression in the hearts of dKO mice changed substantially during disease progression [at 21 d of age (no CAD), 31 d of age (small myocardial infarctions), and 43 d of age (extensive myocardial infarctions) vs. CAD-free SR-BI$^{+/-}$/apoE$^{-/-}$ controls]. Expression of most genes that increased >sixfold in dKO hearts at 43 d also increased after coronary artery ligation. We examined the influence and potential mechanism of action of apolipoprotein D (apoD) whose expression in dKO hearts increased 80-fold by 43 d. Analysis of ischemia/reperfusion-induced myocardial infarction in both apoD KO mice and wild-type mice with abnormally high plasma levels of apoD (adenovirus-mediated hepatic overexpression) established that apoD reduces myocardial infarction. There was a correlation of apoD's ability to protect primary cultured rat cardiomyocytes from hypoxia/reoxygenation injury with its potent ability to inhibit oxidation in a standard antioxidation assay in vitro. We conclude that dKO mice represent a useful mouse model of CAD and apoD may be part of an intrinsic cardioprotective system, possibly as a consequence of its antioxidation activity.

lipocalin | antioxidant

A deeper understanding of the mechanisms underlying coronary artery disease (CAD) and the heart's adaptive and maladaptive responses to it using small animal models of CAD (1) may help inform the development of new approaches for treatment and prevention. The pathogenesis of atherosclerosis in mice is often studied with animals bearing homozygous null apoE or LDL receptor genes, although these mice exhibit little, if any, coronary arterial atherosclerosis, CAD or premature death (2–4), classic hallmarks of human CAD. A mouse model exhibiting many hallmarks of human CAD is a double KO (dKO) mouse with homozygous null mutations in the genes for both apoE and the HDL receptor called scavenger receptor class B, type I (SR-BI) (5–8). When the SR-BI/apoE dKO mice are fed a normal chow diet (low fat), they exhibit severe dyslipidemia (hypercholesterolemia, abnormally large HDL particles with abnormal compositions), occlusive coronary atherosclerosis and thrombosis, and heart failure [cardiomegaly, myocardial infarction (MI), electrocardiographic and echocardiographic abnormalities, and reduced ejection fraction] (5, 6). The CAD culminates in spontaneous death at a mean age of 6 wk (range 5 to 7 or 8 wk, depending on the genetic background; refs. 6, 7, and 9). There is a variant of the dKO mouse (SR-BI KO/ApoeR61$^{h/h}$) whose

disease progression can be adjusted by modifying the severity and timing of administration of the atherogenic diet and is accelerated by social isolation (10, 11). Other mouse models also exhibit robust occlusive coronary arterial atherosclerosis (refs. 12–14; also ref. 15).

The dKO mice have the potential to provide insight into CAD. Some of the complex phenotypes of these mice (e.g., severe dyslipidemia and reticulocytosis) do not mimic those of typical human CAD. These atypical features raise the possibility that their cardiac pathology might not be due simply to occlusive atherosclerosis-based ischemia, thus limiting their usefulness as a model of human CAD. Here, we address this issue by using global mRNA expression profiling of hearts to examine changes in gene expression occurring during CAD development. We analyzed these changes and compared them to those that occur

Significance

Coronary artery disease (CAD) is a major cause of death and disability. Genetically modified SR-BI/apoE double KO (dKO) mice spontaneously exhibit many features of human CAD, including hypercholesterolemia, clogged arteries, myocardial infarction (MI) (or heart attack), heart failure, and premature death. We identified many changes in gene expression in dKO hearts [e.g., increases in apolipoprotein D (apoD)] during CAD development and compared them to those occurring after surgically induced MI. Additional studies showed that apoD partially protected mice from experimentally induced MI (temporarily blocking a coronary artery) and partially protected isolated rat heart muscle cells from temporary oxygen deprivation. We conclude that dKO mice are useful models for human CAD and apoD may naturally help protect hearts from clogged arteries.

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after MI induced by left coronary artery ligation (16, 17). We also focused on the influence and potential mechanism of action of one gene whose expression increased dramatically as disease progressed, apolipoprotein D (apoD). By manipulating the levels of apoD in mice subjected to ischemia/reperfusion (I/R) injury (18), we showed that apoD could be cardioprotective. We found that apoD can protect primary cultured rat cardiomyocytes from hypoxia/reoxygenation (H/R) injury and this protection correlated with and may be influenced by apoD's ability to serve as a potent inhibitor of oxidation in vitro. Induction of cardiac apoD expression may be part of an intrinsic system to protect hearts against damage due to occlusive coronary atherosclerosis.
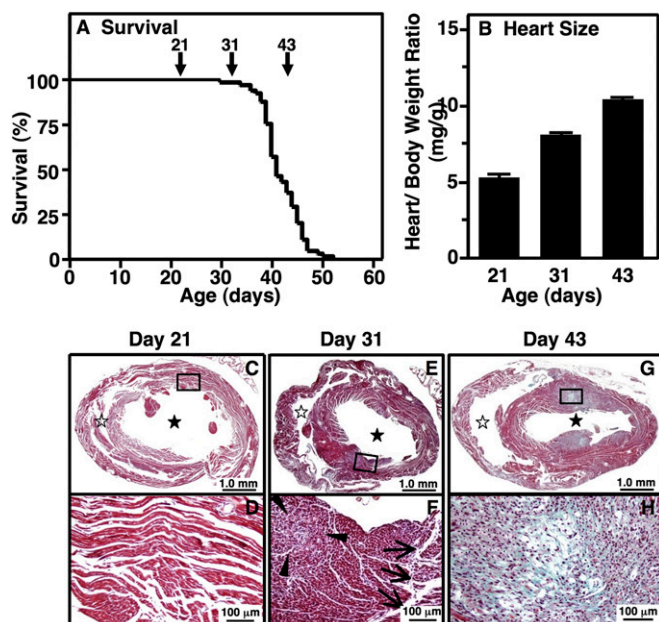
Results

Time Course of Disease Development in $SR-BI^{-/-}/apoE^{-/-}$ Mice. $SR-BI^{-/-}/apoE^{-/-}$ (dKO) mice fed a standard chow diet spontaneously develop occlusive, atherosclerotic CAD and heart failure, with a median survival of ~6 wk (Fig. 1A) (6, 9). In dKO mice, the heart (mg) to body (g) weight ratio was within the normal range (6, 9, 10) at 21 d and increased over time (Fig. 1B), indicating development of heart failure. Histological analysis of hearts in surviving mice showed that at 21 d there were no apparent coronary arterial lesions or MIs (Fig. 1 C and D, no CAD), whereas at 31 d there were atherosclerotic coronary arterial occlusions and small MIs (Fig. 1 E and F, modest disease), and at 43 d there were coronary arterial occlusions and extensive MI (Fig. 1 G and H, severe disease). Control $SR-BI^{+/-}/apoE^{-/-}$ ("Het") mice do not exhibit this pathology (6).To determine how disease development influenced gene expression in the hearts of dKO mice, we sacrificed 9–12 chow-fed dKO or Het mice at 21 d, 31 d, or 43 d of age, extracted mRNA from each heart, and measured gene expression using Affymetrix MG_U74Av2 (12488 probes) gene chips (one heart per chip, 60 hearts analyzed, see *Materials and Methods*);

Fig. 1. Sizes and histological analyses of hearts of dKO mice. dKO mice were maintained on a standard chow diet. (A). Survival curve (data from ref. 9). Arrows indicate ages at which hearts were harvested. (B) Heart (mg) to body weight (g) ratio at 21, 31, and 43 d (n = 10, 10 and 12, respectively) (C–H) Low and high magnification images of Movat pentachrome-stained transverse sections of ventricles. (Scale bar in C, E, and G: 1 mm; and in D, F, and H: 100 μm.) Boxes in C, E, and G indicate the locations of the high-magnification images. The black and white stars indicate the left and right ventricles. The arrows and arrowheads indicate microscopic scarring.

data were deposited in the Gene Expression Omnibus repository under accession number GSE49937.

Microarray Analysis of Gene Expression in dKO and Control Hearts. The microarray data quality is described in the *SI Appendix, Supplemental Results*. Unsupervised clustering using self-organizing maps (*SI Appendix,* Table S1 and *Supplemental Results*) suggests that effects of disease progression on cardiac gene expression in the dKO mice are robustly reproducible. The data sets cluster into five groups that almost perfectly distinguished between hearts from control Het and dKO mice and between hearts of dKO mice at different ages that exhibited different extents of disease progression.

The number of gene probes exhibiting statistically significant expression differences (false discovery rate (FDR) P value <0.05) between dKO and control Het hearts increased dramatically with age (*SI Appendix,* Fig. S1 and *Supplemental Results*). The microarray results for several genes (large and small changes, increases, and decreases) were validated by quantitative RT-PCR (*SI Appendix,* Table S2 and *Supplemental Results*). The largest fold-increase in dKO hearts at 43 d was 416, whereas the largest fold-decrease was 16 (see *SI Appendix,* Table S3 and Datasets S1–S6 for all probes with differences having $P < 0.05$). *SI Appendix,* Fig. S2 shows a heat map (with probes grouped using hierarchical clustering and mice ordered by genotype and age) of those 100 probes, representing 89 independent genes (*SI Appendix, Supplemental Materials and Methods*), exhibiting a >sixfold increase in dKO relative to Het hearts at 43 d. *SI Appendix,* Fig. S3 shows probes with a >twofold decrease at 43 d. Dozens of genes that increased with time in the dKO hearts were previously described to be induced after acute MI (e.g., refs. 16 and 17, and *SI Appendix, Supplemental Results*). As CAD progressed expression increased for inflammation-associated genes and decreased for mitochondrial associated genes, including the transcription factor ERRa (19), as expected (*SI Appendix, Supplemental Results*). *SI Appendix,* Fig. S2 suggests that many of the genes that increased show: (i) relatively little difference in expression levels compared with the Het control at 21 d; (ii) a moderate increase in relative expression at 31 d; and (iii) a striking increase at 43 d. The top two induced genes were osteopontin ("OPN," also called "Spp1," 416-fold increase) and apoD (80-fold increase); their expression levels in dKO mice increased with increasing age (e.g., see *SI Appendix,* Fig. S4A (apoD), *SI Appendix,* Table S2 and Dataset S1). Although cardiac expression of *OPN* previously has been reported explicitly to change in MI (*SI Appendix, Supplemental Results*), this was not the case for apoD (see below).

We compared the 89 genes whose relative expression at 43 d increased >sixfold in dKO hearts to those induced in mouse hearts between 1 h and 8 wk after surgical coronary artery ligation (Cardiogenomics data, refs. 16 and 17). Of these 89 genes, 81 were induced after ligation. The Cardiogenomics data included samples from control, sham operated hearts and from ligated hearts taken from both infarcted and noninfarcted regions. *SI Appendix,* Table S4 and Dataset S7 (also *SI Appendix,* Fig. S4) indicate the temporal and regional distributions of the changes after ligation in expression of those 81 genes, some of which have been reported to increase in the hearts in other models of CAD or in several tissues after pathologic stress. These include genes encoding matricellular proteins, matrix proteases, TIMPs, and inflammation associated proteins (*SI Appendix, Supplemental Results*). Thus, it appears that the largest gene expression changes in the dKO hearts primarily are due to the occlusive CAD in these mice, rather than to the dyslipidemia of dKO mice, as there was no dyslipidemia expected in the coronary artery ligation study (16, 17). The other 8 genes and one poorly annotated probe that increased >sixfold in the dKO data did not increase after coronary artery ligation

([Dataset S7](#) and *SI Appendix*, Fig. S4D). These genes defined a distinct hierarchical cluster of genes (bottom of the heat map in *SI Appendix*, Fig. S2; 7 of which appear to be related to early erythroid development) and presumably are independent of the CAD (see *SI Appendix, Supplemental Results* for additional discussion).
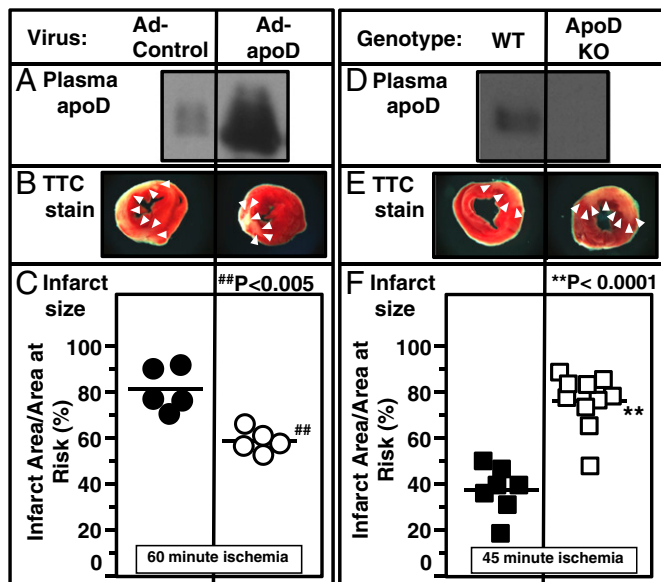
In the ligation experiment, the expression of apoD was distinctive in that its expression was substantially increased in noninfarcted but not infarcted tissue and occurred relatively soon (48 h) after ligation (*SI Appendix*, Fig. S4B and Table S4 and [Dataset S7](#)). Because of apoD's (*i*) dramatic increase in expression in dKO hearts, (*ii*) previously unidentified connection to MI, and (*iii*) distinctively regiospecific expression, we focused subsequent experiments on the possibility that increased apoD expression may be a protective response of intact myocardium (cardiomyocytes, fibroblasts, etc.) to vessel occlusion and consequent ischemic stress.

Effects of Altering apoD Levels on in Vivo Myocardial Infarctions Induced by I/R Injury. apoD previously has been reported to protect against hyperoxic and hypoxic stress in flies and mice and to be induced by neuronal damage (20–24). Here we investigated in mice in vivo the effects on I/R-induced MI of altering apoD expression either by adenovirus (Ad)-mediated, hepatic overexpression (~20-fold increased plasma apoD 4 d after injection, Fig. 2A) or by homozygous genomic knockout (no detectable apoD in the plasma, Fig. 2D).

Effects of adenovirus-mediated apoD overexpression. We performed the ischemia (60 min)/reperfusion (~24 h) procedure 4 d after injection of wild-type (WT) C57BL/6 mice with either an adenovirus encoding mouse apoD (Ad-apoD) or an empty vector control (Ad-control). At the end of the reperfusion period, the ventricles were excised; sections were cut, stained with 2,3,5-triphenyl tetrazolium chloride (TTC), and fixed; and photomicrographs of the sections were used to quantitate the infarcted area (white region, absence of stain) and the area at risk (tissue subjected to ischemia, see *SI Appendix, Supplemental Materials and Methods*). The areas at risk were similar for Ad-control and Ad-apoD–treated mice (~50% of total heart, *SI Appendix*, Fig. S5). Images of representative sections with arrowheads indicating infarcts (noninfarcted areas are red) and the ratios of infarct-area to area-at-risk are shown in Fig. 2 *B* and *C*. The Ad-apoD–induced increase in plasma apoD significantly decreased the relative infarct size (59% vs. 81%, *P* < 0.005), suggesting increased plasma apoD protected the hearts of WT mice.

Effects of apoD KO. Fig. 2 *E* and *F* compare the results of the ischemia (45 min)/reperfusion (~24 h) procedure on WT (Fig. 2 *E* and *F*, *Left*) and apoD KO (Fig. 2 *E* and *F*, *Right*) mice. We observed no significant differences in the plasma levels of cholesterol or triglycerides in apoD KO mice compared with WT controls (*SI Appendix, Supplemental Results*). We use a shorter period of ischemia here than for the adenovirus injected WT mice (60 min) because of increased mortality of apoD KO mice subjected to the longer ischemia. (See *SI Appendix, Supplemental Methods* for additional differences in the experiments with and without adenoviruses.) The areas at risk (~50% of total) were similar for WT and apoD KO mice (*SI Appendix*, Fig. S6). Loss of apoD in the KO mice was associated with a significant increase in infarct area (76% vs. 37%, *P* < 0.0001, Fig. 2 *E* and *F*). Together, I/R studies with apoD KO mice and apoD hepatic overexpression suggest that apoD can be cardioprotective.

Effect of apoD on H/R in Primary Cardiomyocytes. To explore the mechanism of apoD-mediated cardioprotection, we asked whether exogenous apoD protein might directly protect cardiomyocytes against H/R stress in vitro. We subjected primary adult rat ventricular myocytes (ARVMs) or neonatal rat ventricular myocytes (NRVMs) to H/R stress in the absence and presence of 100 µg/mL purified human apoD (or BSA as a control) added to the culture medium. The concentration of apoD added to the culture media was similar to that observed in human plasma (75–300 µg/mL, or ~3–12 µM, see *SI Appendix, Supplemental Methods*).

Fig. 3 shows that for ARVMs H/R stress significantly reduced total cell number (Fig. 3A) and increased the fraction of trypan blue positive staining cells (Fig. 3B) (compare white and hatched bars). Treatment with apoD significantly reversed these effects of H/R (black bars), whereas treatment with BSA did not (gray bars). Treatment with either apoD or BSA of cells not subjected to H/R did not significantly influence total cell number or trypan blue staining. We conclude apoD can protect cultured adult cardiomyocytes against H/R stress. We confirmed these results with NRVMs, quantifying cell viability using the CellTiter-Blue assay. Fig. 4A shows that treatment with 100 µg/mL apoD, but not BSA, significantly reduced (*P* < 0.001) the percent of H/R-induced cell death. It is noteworthy that osteopontin, whose gene expression was the most highly induced in dKO hearts, has also been reported to protect cultured NRVMs from H/R stress (25). Figs. 3 and 4*A* raise the possibility that some of the cardioprotection afforded by apoD in vivo may be a consequence of secreted apoD directly sparing cardiomyocytes from I/R injury.

Mechanism of apoD-Mediated Cardiomyocyte Protection. Little is currently known about the mechanisms by which apoD protects cells and organisms from stress (20–24, 26). One possible mechanism is that as a lipocalin apoD carries a small molecule in its hydrophobic binding pocket [e.g., progesterone (K_d ~ 0.4 µmol/L), arachidonic acid (K_d ~ 0.01 µmol/L) (27–31)] that
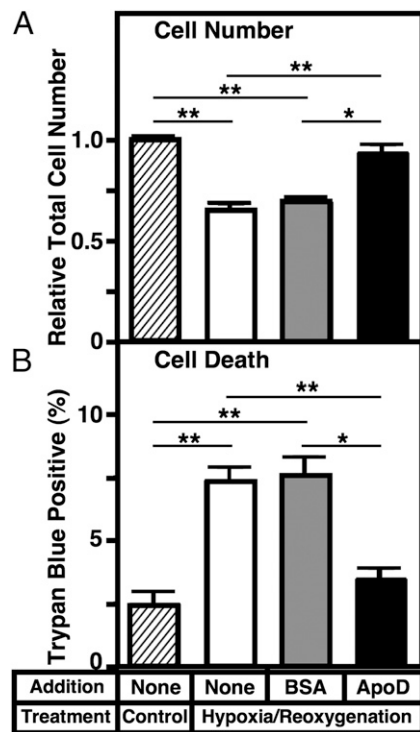

Fig. 2. The effects of hepatic overexpression or null mutations of apoD on plasma apoD (*A* and *D*) and infarct size after in vivo myocardial I/R injury of WT C57BL/6 mice (*B*, *C*, *E*, and *F*). Male mice on a C57BL/6 background were subjected to myocardial ischemia (60 min in *B* and *C* or 45 min in *E* and *F*)/reperfusion (24 h). Fluorescent microspheres were used to visualize the area at risk (AAR) (*SI Appendix*, Figs. S5 and S6). (*A–C*) WT mice (9 wk old) were injected four days before surgery with adenoviruses encoding mouse apoD (Ad-apoD, *n* = 5, *Right*) or an "empty vector" control (Ad-control, *n* = 5, *Left*). (*D–F*) WT (*n* = 7, *Left*) or homozygous null apoD KO (*n* = 10, *Right*) mice (11 wk old) were examined. (*A* and *D*) SDS/PAGE/immunoblotting analysis of plasma apoD (30 µg of protein per lane). In *A*, samples were taken 4 d after virus injection. (*B* and *E*) Representative images of 2,3,5-triphenyl tetrazolium chloride (TTC)-stained sections of hearts. Infarct areas (white) are indicated with arrow heads. (*C* and *F*) Relative infarct sizes (infarct area/AAR) (##*P* < 0.005, **P* < 0.0001).

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Fig. 3. Effect of apoD on H/R stress in cultured adult rat ventricular myocytes (ARVMs). Cultured ARVMs were incubated for 1 h in medium B without ($n = 9$) or with 100 μg/mL of either human apoD ($n = 9$) or BSA ($n = 9$) followed by 16 h of anoxia [95% (vol/vol) N_2 and 5% (vol/vol) CO_2 atmosphere; hypoxia] and then 8 h under normal atmosphere (21% oxygen) (reoxygenation). The apoD or BSA was present as indicated throughout. In addition, control ARVMs were incubated in a normal atmosphere throughout (hatched bars, $n = 8$). At the end of the reoxygenation period, we determined total cell number (*A*) and viability (trypan blue exclusion) (*B*). Error bars represent SEM. (*$P < 0.0005$; **$P < 0.0001$.)

might be protective (20, 21, 27). To determine whether relatively weakly bound ligands were responsible for apoD's protecting cardiomyocytes, we extensively dialyzed human apoD and examined

its activity in the NRVM-H/R CellTiter-Blue assay. Fig. 4*B* shows dialysis did not significantly reduce apoD's activity, and thus, it is unlikely that a relatively weakly bound ligand is responsible for its activity. To assess the role of apoD's conformation on activity, we denatured apoD by boiling (100 °C, ≥5 min) before the assay. Fig. 4*B* shows that boiling eliminated virtually all of apoD's activity. We conclude that the properly folded conformation of apoD is essential for its cardiomyocyte protective activity.

Previous reports have suggested that some physiologic activities of apoD may involve antioxidant activity (reviewed in ref. 21, also refs. 24, 26, 28, 32, 33). Thus, we compared the abilities of apoD and the control antioxidant Trolox (a water-soluble derivative of vitamin E) to inhibit the oxidation of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) by hydrogen peroxide and myoglobin (34). Fig. 4*C* shows that apoD exhibited a very potent antioxidant activity: 1.2 μmol/L apoD was equivalent to 314 μmol/L Trolox. Extensive dialysis did not, but boiling did (71%), inhibit apoD's antioxidant activity in this assay (Fig. 4*C*). We conclude that the properly folded conformation of apoD, but not a relatively weakly bound ligand, is essential for its antioxidation activity in this assay. The correlation of apoD's antioxidant and cardiomyocyte protective activities suggest this activity is a potential mechanism for apoD's cardioprotective activity. Future studies will be required to determine whether this is the case.

Discussion

In the current study, we have identified many genes whose expression in hearts is significantly altered as a function of age in the dKO model of occlusive coronary arterial atherosclerotic heart disease (*SI Appendix*, Table S3 and Datasets S1–S6) (5–7, 9). There was a striking concordance of those genes whose expression increased most with those previously observed to increase in ligation-induced MI (refs. 16 and 17, and *SI Appendix, Supplemental Results*). Thus, the dKO mouse appears to be a useful animal model for identifying mechanisms underlying the pathology of CAD and the heart's endogenous protective responses to injury.

Here, we have focused on apoD, one of the genes whose expression changed dramatically with time in the dKO hearts but
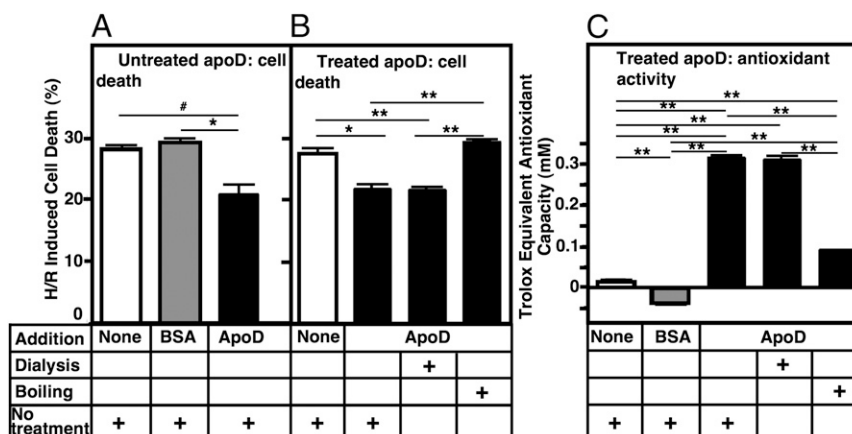
Fig. 4. Effects of untreated, dialyzed or boiled apoD on H/R stress in cultured neonatal rat ventricular myocytes (NRVMs) and in vitro oxidation of ABTS. (*A* and *B*) NRVMs ($n = 8$ for each condition) were preincubated for 1 h in medium B without or with 100 μg/mL of either untreated, extensively dialyzed or boiled (5 min) human apoD or BSA followed by 24 h of hypoxia [95% (vol/vol) N_2 and 5% (vol/vol) CO_2 atmosphere] and then 6 h of reoxygenation (normal atmosphere). The apoD or BSA was present as indicated throughout. Control NRVMs also were incubated in a normal atmosphere throughout. At the end of the reoxygenation period, relative cell viability in each well was assessed using the CellTiter Blue method, and presented as H/R induced cell death (% of cells, see *Materials and Methods*). (*C*) In vitro antioxidant capacities of 30 μg/mL of either untreated, extensively dialyzed, or boiled (≥5 min) human apoD or BSA were measured using the ABTS assay (see *Materials and Methods*, $n = 8$ for each condition). Antioxidant capacity is presented as Trolox equivalents. Error bars represent SEM. (#$P < 0.001$; *$P < 0.0005$; **$P < 0.0001$.)

had not previously been studied in the context of MI. Future studies will be required to determine the cell types in dKO hearts in which the apoD expression was induced (cardiomyocytes, fibroblasts, etc.) and the mechanisms responsible for the induction. apoD (reviewed in refs. 20 and 21) is a secreted ~25- to 29-kDa glycoprotein (associated with HDL in the plasma) and a member of the lipocalin family (β-barrel structure with elongated hydrophobic pocket that binds small molecule ligands) (27–31). It is expressed highly in nervous tissue and apparently at lower levels in other tissues under normal conditions. apoD expression is increased with aging, in neurological and psychiatric disorders, and in some cancers (35, 36). Studies in apoD KO mice and mice overexpressing a human or murine apoD transgene indicate that apoD is required for normal triglyceride metabolism (37, 38), neuronal function (21), and adequate and timely response to injury (23), and protects against oxidative stress (21, 33). There are apoD homologs in flies and plants whose physiologic roles appear to parallel those in mammals (e.g., see ref. 21). In a brief report, Wei et al. (39) noted a three- to sixfold increase in apoD mRNA expression in the hearts of a few patients with various cardiomyopathies (e.g., dilated and hypertrophic cardiomyopathy) accompanied by increased apoD protein detected in myocardial cells by immunohistochemistry.

Using previously reported gene expression data (16, 17), we observed that apoD expression is induced in mouse hearts at relatively early times after coronary artery ligation, primarily in noninfarcted regions. Because of apoD's increased expression in CAD and ability to protect against multiple stresses (20–23), we determined whether apoD were cardioprotective in an I/R surgical model of MI in mice (18). Indeed, adenovirus-mediated hepatic overexpression of apoD reduced and genetic ablation of apoD (apoD KO) increased MI relative to controls. Thus, apoD exhibited potent cardioprotective activity in vivo, suggesting that the increased apoD expression in the hearts of dKO mice with progression of CAD may be part of a system to protect hearts against damage due to ischemia.

We explored the potential mechanism underlying apoD's cardioprotective activity and found that purified human apoD added to the culture medium could specifically and effectively protect cultured primary rat cadiomyocytes from H/R injury. Thus, some of the cardioprotection afforded by apoD in vivo may be a consequence of apoD's directly preventing cardiomyocyte injury. apoD's cardiomyocyte protective activity in vitro is unlikely to be due to its carrying a relatively low affinity binding bioactive small molecule (activity resistant to extensive dialysis), but does appear to depend on its conformation (boiling sensitive) and is correlated with its in vitro inhibition of a standard oxidation assay (34). This correlation raises the possibility that apoD might be cardioprotective because it is an antioxidant. In future studies, it will be instructive to determine the effects of apoD on reactive oxygen species generation in cardiomyocytes and the ability of other antioxidants to rescue the enhanced I/R injury in apoD KO mice. Independently of our work, Bhatia et al. (24, 28) reported that mammalian apoD could function in vitro as an antioxidant in a free-radical-generating lipid hydroperoxide assay. They proposed that one methionine in native apoD significantly contributes to its antioxidant activity. Previous studies have suggested that apoD may function directly or indirectly as an antioxidant in vivo in mice and *Drosophila melanogaster* (21, 32). The apoD from a fish-like marine protochordate and other lipocalins also exhibit antioxidant activities (26, 40, 41). The relationship between apoD's antioxidant activities in the ABTS/urea hydrogen peroxide/myoglobin assay and in the lipid hydroperoxide assay (24) remains to be determined. There are alternative potential mechanisms of the cardioprotective activity of apoD. For example, it has been proposed that apoD can function as a signaling molecule to alter activities of target cells (42). It is also possible that apoD may sequester toxic small molecules generated during stress.

Protection of cultured primary cardiomyocytes from H/R injury in serum-free medium suggests that the cardioprotective activity of apoD in vivo may be due to its direct effects on cardiomyocytes that may be independent of plasma proteins or lipoproteins. However, it remains possible that some of the apoD-mediated cardioprotection may be due to effects on other types of cells in the heart (e.g., fibroblasts, vascular cells) or be indirect due to systemic effects of apoD on other tissues or on the structure and/or function of the lipoproteins that carry most of the apoD in the plasma. Future studies will be required to determine precisely the mechanism of apoD's cardioprotective activity in vivo and if the identification of apparently cardioprotective genes such as apoD will lead to new approaches for the diagnosis and treatment of CAD.

Materials and Methods

A detailed description of the materials and methods used in this study may be found in the *SI Appendix*. These methods include: generation of apoD KO mice, histological analysis, RNA isolation, microarray analysis, quantitative RT-PCR, gel electrophoresis and immunoblotting, human apoD purification, dialysis and heat denaturation, antioxidant assay, isolation of primary rat cardiomyocytes, the cardiomyocyte H/R procedure, adenovirus-mediated gene transfer, I/R surgery, and histological evaluation of MI. Experiments using animals were performed in accordance with the guidelines of the Committee on Animal Care at the Massachusetts Institute of Technology. P values < 0.05 are considered significant. Except for P values associated with FDR for the microarray analysis (*SI Appendix*), all P values (Student's t test) and SEM values were calculated using Prism 5 software (GraphPad).

1. Zaragoza C, et al. (2011) Animal models of cardiovascular diseases. *J Biomed Biotechnol* 2011:497841.
2. Plump AS, et al. (1992) Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell* 71(2):343–353.
3. Zhang SH, Reddick RL, Piedrahita JA, Maeda N (1992) Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science* 258(5081):468–471.
4. Ishibashi S, et al. (1993) Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. *J Clin Invest* 92(2):883–893.
5. Trigatti B, et al. (1999) Influence of the high density lipoprotein receptor SR-BI on reproductive and cardiovascular pathophysiology. *Proc Natl Acad Sci USA* 96(16):9322–9327.
6. Braun A, et al. (2002) Loss of SR-BI expression leads to the early onset of occlusive atherosclerotic coronary artery disease, spontaneous myocardial infarctions, severe cardiac dysfunction, and premature death in apolipoprotein E-deficient mice. *Circ Res* 90(3):270–276.
7. Braun A, et al. (2003) Probucol prevents early coronary heart disease and death in the high-density lipoprotein receptor SR-BI/apolipoprotein E double knockout mouse. *Proc Natl Acad Sci USA* 100(12):7283–7288.
8. Rigotti A, Miettinen HE, Krieger M (2003) The role of the high-density lipoprotein receptor SR-BI in the lipid metabolism of endocrine and other tissues. *Endocr Rev* 24(3):357–387.
9. Karackattu SL, Picard MH, Krieger M (2005) Lymphocytes are not required for the rapid onset of coronary heart disease in SR-BI/apoE double knockout mice. *Arterioscler Thromb Vasc Biol* 25(4):803–808.
10. Zhang S, et al. (2005) Diet-induced occlusive coronary atherosclerosis, myocardial infarction, cardiac dysfunction, and premature death in scavenger receptor class B type I-deficient, hypomorphic apolipoprotein ER61 mice. *Circulation* 111(25):3457–3464.
11. Nakagawa-Toyama Y, Zhang S, Krieger M (2012) Dietary manipulation and social isolation alter disease progression in a murine model of coronary heart disease. *PLoS ONE* 7(10):e47965.

MEDICAL SCIENCES

12. Caligiuri G, Levy B, Pernow J, Thorén P, Hansson GK (1999) Myocardial infarction mediated by endothelin receptor signaling in hypercholesterolemic mice. *Proc Natl Acad Sci USA* 96(12):6920–6924.

13. Cozen AE, et al. (2004) Macrophage-targeted overexpression of urokinase causes accelerated atherosclerosis, coronary artery occlusions, and premature death. *Circulation* 109(17):2129–2135.

14. Fernández-Hernando C, et al. (2007) Loss of Akt1 leads to severe atherosclerosis and occlusive coronary artery disease. *Cell Metab* 6(6):446–457.

15. Nakata S, et al. (2008) Spontaneous myocardial infarction in mice lacking all nitric oxide synthase isoforms. *Circulation* 117(17):2211–2223.

16. Tarnavski O, et al. (2004) Mouse cardiac surgery: Comprehensive techniques for the generation of mouse models of human diseases and their application for genomic studies. *Physiol Genomics* 16(3):349–360.

17. NHLBI Program for Genomic Applications, Harvard Medical School (2003) Genomics of Cardiovascular Development, Adaptation, and Remodeling. Available at www.cardiogenomics.org. Accessed October 21, 2009.

18. Jain M, et al. (2001) Cell therapy attenuates deleterious ventricular remodeling and improves cardiac performance after myocardial infarction. *Circulation* 103(14): 1920–1927.

19. Karamanlidis G, et al. (2010) Defective DNA replication impairs mitochondrial biogenesis in human failing hearts. *Circ Res* 106(9):1541–1548.

20. Perdomo G, Henry Dong H (2009) Apolipoprotein D in lipid metabolism and its functional implication in atherosclerosis and aging. *Aging (Albany, NY Online)* 1(1): 17–27.

21. Muffat J, Walker DW (2010) Apolipoprotein D: An overview of its role in aging and age-related diseases. *Cell Cycle* 9(2):269–273.

22. Do Carmo S, Jacomy H, Talbot PJ, Rassart E (2008) Neuroprotective effect of apolipoprotein D against human coronavirus OC43-induced encephalitis in mice. *J Neurosci* 28(41):10330–10338.

23. Ganfornina MD, et al. (2010) ApoD, a glia-derived apolipoprotein, is required for peripheral nerve functional integrity and a timely response to injury. *Glia* 58(11): 1320–1334.

24. Bhatia S, et al. (2012) Selective reduction of hydroperoxyeicosatetraenoic acids to their hydroxy derivatives by apolipoprotein D: Implications for lipid antioxidant activity and Alzheimer's disease. *Biochem J* 442(3):713–721.

25. Wang Y, Chen B, Shen D, Xue S (2009) Osteopontin protects against cardiac ischemia-reperfusion injury through late preconditioning. *Heart Vessels* 24(2):116–123.

26. Zhang Y, Cong Y, Wang S, Zhang S (2011) Antioxidant activities of recombinant amphioxus (Branchiostoma belcheri) apolipoprotein D. *Mol Biol Rep* 38(3):1847–1851.

27. Eichinger A, Nasreen A, Kim HJ, Skerra A (2007) Structural insight into the dual ligand specificity and mode of high density lipoprotein association of apolipoprotein D. *J Biol Chem* 282(42):31068–31075.

28. Oakley AJ, Bhatia S, Ecroyd H, Garner B (2012) Molecular dynamics analysis of apolipoprotein-D-lipid hydroperoxide interactions: Mechanism for selective oxidation of Met-93. *PLoS ONE* 7(3):e34057.

29. Pearlman WH, Guériguian JL, Sawyer ME (1973) A specific progesterone-binding component of human breast cyst fluid. *J Biol Chem* 248(16):5736–5741.

30. Dilley WG, Haagensen DE, Cox CE, Wells SA, Jr. (1990) Immunologic and steroid binding properties of the GCDFP-24 protein isolated from human breast gross cystic disease fluid. *Breast Cancer Res Treat* 16(3):253–260.

31. Morais Cabral JH, et al. (1995) Arachidonic acid binds to apolipoprotein D: Implications for the protein's function. *FEBS Lett* 366(1):53–56.

32. Bajo-Grañeras R, Ganfornina MD, Martín-Tejedor E, Sanchez D (2011) Apolipoprotein D mediates autocrine protection of astrocytes and controls their reactivity level, contributing to the functional maintenance of paraquat-challenged dopaminergic systems. *Glia* 59(10):1551–1566.

33. Walker DW, Muffat J, Rundel C, Benzer S (2006) Overexpression of a Drosophila homolog of apolipoprotein D leads to increased stress resistance and extended lifespan. *Curr Biol* 16(7):674–679.

34. Miller NJ, Rice-Evans CA (1997) Factors influencing the antioxidant activity determined by the ABTS.+ radical cation assay. *Free Radic Res* 26(3):195–199.

35. Aspinall JO, et al. (1995) Differential expression of apolipoprotein-D and prostate specific antigen in benign and malignant prostate tissues. *J Urol* 154(2 Pt 1):622–628.

36. Blais Y, et al. (1995) Interleukin-6 inhibits the potent stimulatory action of androgens, glucocorticoids and interleukin-1 alpha on apolipoprotein D and GCDFP-15 expression in human breast cancer cells. *Int J Cancer* 62(6):732–737.

37. Perdomo G, et al. (2010) A role of apolipoprotein D in triglyceride metabolism. *J Lipid Res* 51(6):1298–1311.

38. Jiménez-Palomares M, Cózar-Castellano I, Ganfornina MD, Sánchez D, Perdomo G (2011) Genetic deficiency of apolipoprotein D in the mouse is associated with non-fasting hypertriglyceridemia and hyperinsulinemia. *Metabolism* 60(12):1767–1774.

39. Wei YJ, et al. (2008) Apolipoprotein D as a novel marker in human end-stage heart failure: A preliminary study. *Biomarkers* 13(5):535–548.

40. Roudkenar MH, et al. (2011) Neutrophil gelatinase-associated lipocalin: A new antioxidant that exerts its cytoprotective effect independent on Heme Oxygenase-1. *Free Radic Res* 45(7):810–819.

41. Olsson MG, et al. (2011) Up-regulation of A1M/α1-microglobulin in skin by heme and reactive oxygen species gives protection from oxidative damage. *PLoS ONE* 6(11): e27505.

42. Leung WC, et al. (2004) Apolipoprotein D and platelet-derived growth factor-BB synergism mediates vascular smooth muscle cell migration. *Circ Res* 95(2):179–186.