

Reduced antioxidant capacity and diet-induced atherosclerosis in uncoupling protein-2-deficient mice^S

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Abstract Vascular dysfunction in response to reactive oxygen species (ROS) plays an important role in the development and progression of atherosclerotic lesions. In most cells, mitochondria are the major source of cellular ROS during aerobic respiration. Under most conditions the rates of ROS formation and elimination are balanced through mechanisms that sense relative ROS levels. However, a chronic imbalance in redox homeostasis is believed to contribute to various chronic diseases, including atherosclerosis. Uncoupling protein-2 (UCP2) is a mitochondrial inner membrane protein shown to be a negative regulator of macrophage ROS production. In response to a cholesterol-containing atherogenic diet, C57BL/6J mice significantly increased expression of UCP2 in the aorta, while mice lacking UCP2, in the absence of any other genetic modification, displayed significant endothelial dysfunction following the atherogenic diet. Compared with wild-type mice, *Ucp2*^{-/-} mice had decreased endothelial nitric oxide synthase, an increase in vascular cell adhesion molecule-1 expression, increased ROS production, and an impaired ability to increase total antioxidant capacity. These changes in *Ucp2*^{-/-} mice were associated with increased aortic macrophage infiltration and more numerous and larger atherosclerotic lesions. These data establish that in the vasculature UCP2 functions as an adaptive antioxidant defense to protect against the development of atherosclerosis in response to a fat and cholesterol diet.—Moukdar, F., J. Robidoux, O. Lyght, J. Pi, K. W. Daniel, and S. Collins. **Reduced antioxidant capacity and diet-induced atherosclerosis in uncoupling protein-2-deficient mice.** *J. Lipid Res.* 2009. 50: 59–70.

Supplementary key words UCP2 • reactive oxygen species • ROS • inflammation

Atherosclerosis is a multifactorial chronic vascular disease whose prevalence is increasing worldwide approaching epidemic proportions (1). It is believed that atherosclerosis

is initiated by a combination of systemic and local inflammatory events that promote all phases of plaque development and progression (2). Moreover, studies using animal models of atherosclerosis have documented that reactive oxygen species (ROS), which are produced and used by all plaque constituents, serve as one of the drivers of the atherosclerotic process (as reviewed in Refs. 3, 4). Indeed, lesion formation is associated with a collection of events that are regulated by ROS: accumulation of lipid peroxidation products (5, 6), induction of inflammatory/inflammation-related genes (7), inactivation of nitric oxide (NO) leading to endothelial dysfunction (8, 9), activation of matrix metalloproteinases (10), and increased smooth muscle cell growth (11).

As a defense against oxidative stress, most eukaryotic cells are equipped with enzymatic and nonenzymatic mechanisms to neutralize oxidants. These mechanisms have been studied extensively in the heart, and the most important of these include enzymes such as superoxide dismutases (SOD), catalase, and glutathione peroxidase (GPx) (12–15). Under normal conditions, ROS and reactive nitrogen species (RNS) are generated as byproducts of oxidative metabolic activity, but are now appreciated to also serve as signaling molecules in some settings (16–19). However, under pathophysiological conditions, persistently high levels of ROS/RNS can outstrip endogenous antioxidant defense systems resulting in oxidation of biological molecules such as DNA, proteins, and lipids (20). Thus, maintaining a balance between formation and elimination of ROS is necessary for their optimal performance in signaling and scavenging functions (16, 19).

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One of the factors receiving increased attention as a regulator of ROS is uncoupling protein-2 (UCP2). UCP2 was originally discovered as a structural homolog of the brown fat UCP1 (21). Although UCP2 was proposed to function in adaptive thermogenesis in a manner equivalent to UCP1, it now appears that UCP2 primarily acts to dampen ROS generation. The exact biochemistry of this process is still under debate, but UCP2 has been shown to decrease mitochondrial ROS production in a number of cell types and organs (22–26). From a clinical perspective, it is interesting that a common polymorphism in the human UCP2 gene has been associated with low levels of UCP2 expression and a number of cardiovascular risk factors (27), including asymptomatic carotid atherosclerosis in women (28) and low density lipoprotein particle size (29).

The first report describing *Ucp2*^{-/-} mice revealed that they exhibited an increased macrophage inflammatory profile (22). Further studies showed that these macrophages produce significantly more cytokines and NO due to a constitutively activated NFκB system (30). To determine if these macrophages from *Ucp2*^{-/-} mice might influence the size or progression of atherosclerotic plaque development in the atherosclerosis-prone LDLR^{-/-} mouse, Blanc et al. (24) showed that bone marrow transplantation from donor *Ucp2*^{-/-} mice into the mutant LDLR recipient led to increased plaque size. Apart from macrophages, studies in cultured endothelial cells have shown that forced overexpression of UCP2 can inhibit ROS production (31). Other observed effects of UCP2 included inhibiting vascular smooth muscle cell proliferation and migration into the intima (32), and diminished monocyte accumulation in the arterial wall by inhibiting both their firm adhesion and transendothelial migration (33). Since we and others have shown that the absence of UCP2 in mice is associated with elevated oxidative stress, it is thus reasonable to suspect that the absence of UCP2, in multiple cells types that are together involved in the development of the atherosclerotic plaque, might render the animals more susceptible to atherogenic environmental conditions. Therefore we proposed that *Ucp2*^{-/-} mice per se might develop aortic lesions, even in the absence of any other single gene deletion such as LDLR that is already predisposed to the condition. In this study we investigated the impact of UCP2 on antioxidant status and the development of atherosclerosis in *Ucp2*^{+/+} vs. *Ucp2*^{-/-} mice that have been extensively backcrossed into the C57BL/6J background (J. Pi et al., unpublished observations).

EXPERIMENTAL PROCEDURES

Chemicals and diets

Oil Red O and pentobarbital were from Sigma Aldrich (Milwaukee, WI). Hematoxylin and OCT embedding medium were from Fisher (Santa Clara, CA). The atherogenic and control diets were from Harlan Teklad (Madison, WI). The atherogenic diet (TD88051) contained approximately, in percentage per weight, 15.8% fat, 1.25% cholesterol, and 0.5% sodium cholate and the control diet (TD95138) was an isocaloric diet with no

added cholesterol or cholate. The composition of these diets is based on the work of Paigen et al. (34).

Animals

Female C57BL/6J wild-type (*Ucp2*^{+/+}) and *Ucp2*^{-/-} mice from our colony were used in this study. Generation of *Ucp2*^{-/-} mice was described previously (22). The animals in this study had been backcrossed from the mixed C57Bl/6J-129Sv/J strain background to C57BL/6J mice (JAX stock #000664) for more than 12 generations. All studies were performed using age-matched wild-type and *Ucp2*^{-/-} mice, and were approved by the Institutional Animal Care and Use Committee of The Hamner Institutes for Health Sciences.

After weaning at 3–4 weeks of age, the mice were housed in groups of three and fed a Rodent diet NIH-07 (Zeigler Brothers, Gardner, PA) for 1 week, at which time they were switched to either a control diet (TD95138) or an atherogenic diet (TD88051) for 14 weeks (34). All animals had free access to food and water. Their body weights and food intake were monitored twice per week.

Blood collection and biochemical assays

At the end of the 14-week treatment the mice were anesthetized (pentobarbital, 45 mg/Kg; i.p) and a blood sample was collected by retroorbital sinus puncture. An aliquot of whole blood was kept for glutathione measurement and the remainder was centrifuged 10 min at 12,000 *g* for plasma harvesting. The following plasma measurements were made: total and HDL cholesterol (Wako: #439-17501 and #431-52501); triglycerides (Sigma Aldrich: T2449); adiponectin, leptin, and resistin (R and D ELISA kits: MRP300, MOB00, MRSN00, respectively); insulin (Linco: #EZRMI-13K); CRP (ALPCO: #41-CRPP-5E); SAA (BioSource: #KMA0011); and glucose was evaluated with a glucometer (Roche, Accu-Chek Aviva). Total and oxidized glutathione levels were measured using a kit from OxisResearch (#21040). The antioxidant activity was measured as the capacity of the plasma to inhibit the production of thiobarbituric acid reactive substances (TBARS) from sodium benzoate as described by Koracevic et al. (35). The enzymatic activity of GPx and catalase were measured using kits from Sigma-Aldrich (CGP-1 and CAT100, respectively), and superoxide dismutase activity was measured using a kit from Fluka (#19160). Detection of cytokines was performed using the Bio-Plex Mouse Cytokine system from BioRad Laboratories (Hercules, CA).

Analysis of fatty streak lesion

After 14 weeks on the atherogenic or control diet, the animals were anesthetized with pentobarbital (45 mg/Kg; ip). The heart was perfused with PBS to remove blood, and then the heart and the upper section of the aorta were removed. The lower portion of the heart was removed with the plane of sectioning parallel to a line between the tips of the atria, and the remaining was imbedded in OCT compound, frozen and kept at -70°C for lesion analysis. Aortic sections were prepared according to the method described by Paigen et al. (36). Sections were stained with Oil Red O and counterstained with hematoxylin. The Oil Red O stained areas in the aortic wall were measured and the average number and size of lesions/mouse determined using a computer assisted-video imaging system (Image-Pro Plus 5.0, MediaCybernetics).

Immunohistochemistry

All immunostaining procedures were performed on frozen sections. Macrophages were detected with the anti-mouse F4/80 (diluted 1:100, Serotec). Endothelial cells were identified by the antibody against Von Willebrand Factor (dilution 1:200, DAKO cytometry). Nitrotyrosine was detected using the anti-mouse anti-nitrotyrosine (dilution 1:20, Upstate). In each case, sections

were incubated with the specific antibody for 60 min, then washed for 5 min with PBS and incubated for 60 min with a polyclonal biotinylated secondary antibody (Vector Laboratories). Slides were washed with PBS, incubated with streptavidin-HRP (dilution: 1:50, Zymed Laboratories), and peroxidase activity was detected with the AEC kit (Zymed Laboratories). Sections were counterstained with Gill's modified hematoxylin (Dako cytomation).

RNA isolation, reverse transcriptase-PCR, and real-time PCR

Total RNA was extracted from harvested tissues using TriReagent (Sigma-aldrich), and cDNA was generated using the High Capacity cDNA Archive kit from Applied Biosystems following their protocol, which was scaled down to a 50 μ l total volume. Real-time PCR was performed using TaqMan Primers and probes mixes from Applied Biosystems (Foster City, CA) on an ABI PRISM 7700 Sequence Detector from Perkin Elmer (Boston, MA) following the manufacturer protocol. GAPDH was used as the internal standard.

H₂O₂ Measurement

The quantification of H₂O₂ in the frozen sections was performed using the Amplex Red Hydrogen Peroxide-Peroxidase Assay (Invitrogen-Molecular Probes). Absorbance was measured at 590 nm with a plate reader (Victor 3, Perkin Elmer). The concentration of H₂O₂ in each sample was calculated from a standard curve.

Statistical analysis

Data are expressed as mean \pm SEM. Two-way ANOVA followed by a Tukey's or Dunnett's multiple comparisons test was used to compare treated groups with controls. A *P* value of < 0.05 was considered significant.

RESULTS

Development of atherosclerotic lesions in *Ucp2*^{-/-} mice fed an atherogenic diet

To test the hypothesis that UCP2 may be protective against diet-induced atherosclerotic lesions, female congenic C57BL/6J (B6) *Ucp2*^{+/+} and *Ucp2*^{-/-} mice were fed either a control or atherogenic diet developed by Paigen et al. (34) for 14 weeks. First we examined the expression of UCP2 in wild-type mice following the atherogenic diet regimen. As shown in **Fig. 1**, there was a nearly 4-fold increase in *Ucp2* expression in the aorta of wild-type mice. This finding is consistent with previous observations in other tissues, that the expression of UCP2 is increased in response to conditions of oxidative stress or inflammation (37–39). Histological examination of sections from the aortic sinus region was performed. As shown in **Fig. 2A** Oil Red O-positive cells were evident in the intimal space between the endothelial cells and the subadjacent smooth muscle cell layer of both genotypes fed the atherogenic diet, with no neutral lipid accumulation detected in samples from either genotype fed the control diet. Based on the quantification of the Oil Red O stained areas, morphometric analysis of the lesions in the aortic sinus revealed a \sim 5-fold increase in lipid deposition in the *Ucp2*^{-/-} mice compared with a \sim 2-fold increase in the *Ucp2*^{+/+} mice (**Fig. 2B**). Not only were the lesions in

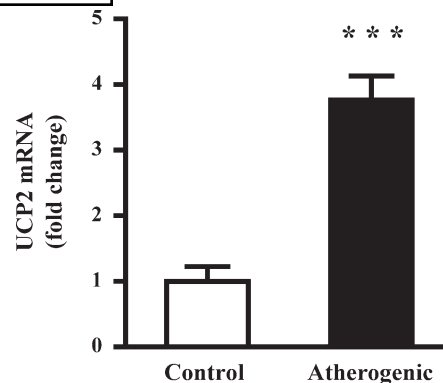


Fig. 1. Uncoupling protein-2 (UCP2) expression in wild-type (*Ucp2*^{+/+}) mice fed the control or atherogenic diet for 14 weeks. Bars indicate the effect of diet on *Ucp2* expression level in the aorta. Results are presented as mean \pm SEM; *n* = 8–12 in each group; *** *P* < 0.001.

the *Ucp2*^{-/-} mice significantly larger, they were also more numerous (**Fig. 2C**).

Body weights and plasma parameters

As shown in **Table 1** and supplemental Fig. I, the wild-type and *Ucp2*-null mice displayed similar body weight gains during the 14 weeks on the atherogenic diet. Although both *Ucp2*^{+/+} and *Ucp2*^{-/-} mice developed significant hypercholesterolemia, there was no significant change in either HDL-C or plasma triglycerides (**Table 1**). Adiponectin has been reported to be an anti-inflammatory (40) and antiatherogenic adipokine (41, 42). Also shown in **Table 1**, both genotypes displayed the same significant decrease in adiponectin when fed the atherogenic diet. By contrast, plasma levels of serum amyloid A (SAA) were significantly more elevated in *Ucp2*-null mice even under basal conditions. This is important because SAA can increase considerably in response to inflammatory challenge (43, 44), and arterial accumulation correlates significantly with lesion area (45). After 14 weeks on the atherogenic diet, both genotypes showed a significant increase in SAA levels. However, note that this increase in *Ucp2*^{+/+} mice was equivalent to the already elevated basal level of *Ucp2*^{-/-} mice, which showed an even further significant rise in response to diet.

Heightened local inflammation in the aortic sinus of *Ucp2*^{-/-} mice

An elevated macrophage inflammatory response was the first phenotype to be characterized for mice lacking UCP2 (22). Because the infiltration of monocytes/macrophages into the lesion is a hallmark of atherosclerosis, we examined the aortae of wild-type and *Ucp2*^{-/-} mice by immunostaining for F4/80. As shown in **Fig. 3A**, F4/80 levels were already higher in UCP2-deficient mice consuming the control diet than in wild-type mice. After 14 weeks on the atherogenic diet, the immunostained areas were significantly greater in UCP2-deficient mice compared with the wild-type, and they tended to be localized to the plaques

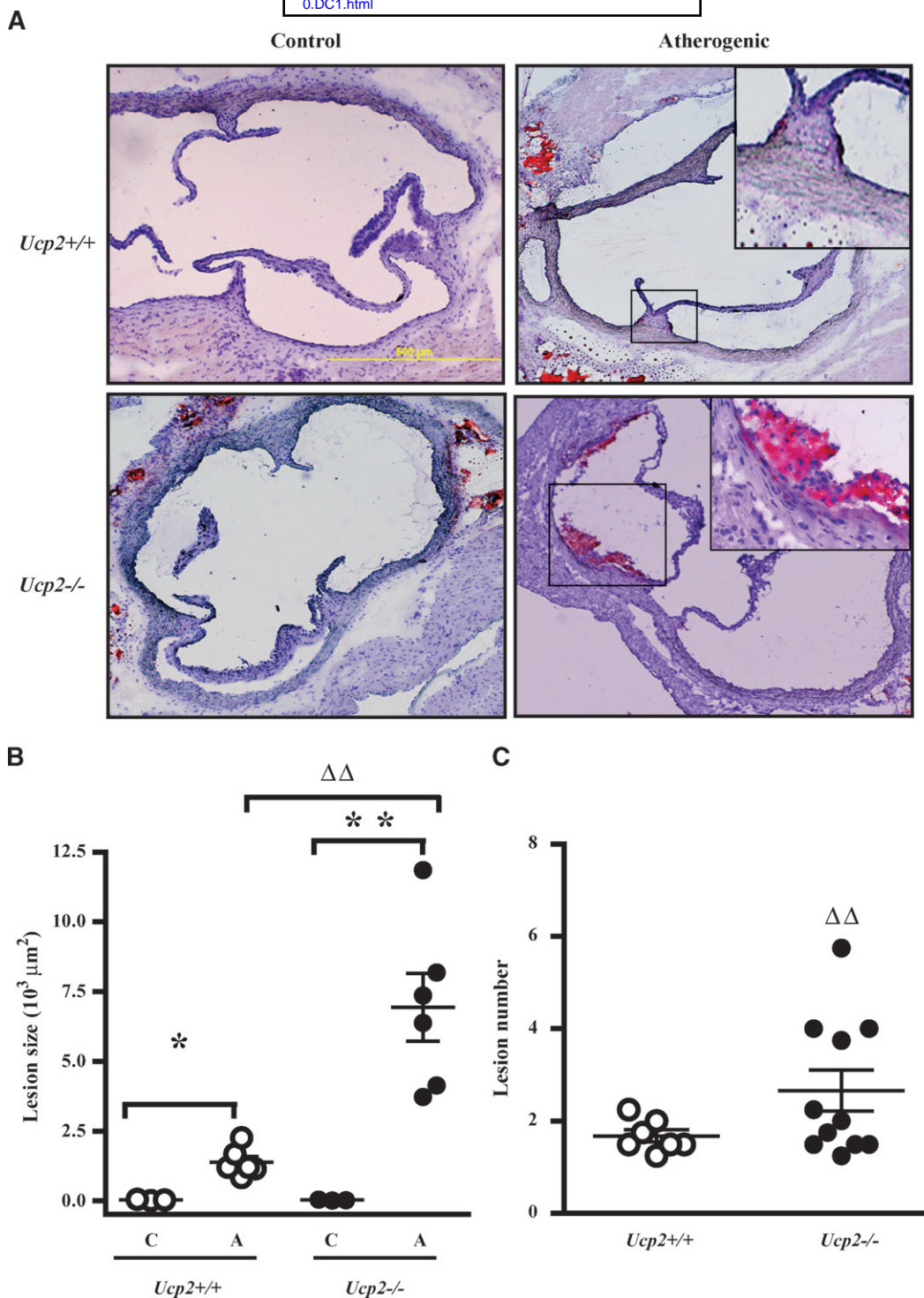


Fig. 2. Increased development of atherosclerotic lesions in the aortic sinus of *Ucp2*^{-/-} mice fed atherogenic diet. A: Representative aortic sections from female congenic C57BL/6J (B6) *Ucp2*^{+/+} and *Ucp2*^{-/-} mice on control or atherogenic diets for 14 weeks. Sections were stained with Oil Red O and hematoxylin as described in Methods. Original magnification $\times 100$. B: Aortic lesion size (area) and number in *Ucp2*^{+/+} (○) and *Ucp2*^{-/-} (●) mice on control (C) or atherogenic (A) diets. Results are presented as mean \pm SEM; n = 6–10 per group. * $P < 0.01$, ** $P < 0.001$ when comparing effect of diet within same genotype; $\Delta\Delta$ $P < 0.001$, when comparing effect of genotype within the same diet. Data for lesion number are for the atherogenic diet.

coincident with the Oil Red O accumulation. Consistent with these morphological measurements, levels of F4/80 mRNA measured in the aortic wall showed an equivalent pattern (Fig. 3B). As another measure of local inflamma-

tory status, we also examined the expression of IL-6 in the aorta. Fig. 3C shows that in wild-type mice there was a small but insignificant increase in response to the atherogenic diet. However, in *Ucp2*-null mice IL-6 expression was

TABLE 1. Body weight and biochemical parameters of female congenic C57BL/6J (B6) *Ucp2*^{+/+} and *Ucp2*^{-/-} mice fed control or atherogenic diet for 14 weeks

	<i>Ucp2</i> ^{+/+} Control diet	<i>Ucp2</i> ^{+/+} Atherogenic diet	<i>Ucp2</i> ^{-/-} Control diet	<i>Ucp2</i> ^{-/-} Atherogenic diet
Weight gain (g)	7.34 ± 0.55	4.96 ± 0.49 ^a	6.25 ± 0.45	5.08 ± 0.34 ^b
Total cholesterol (mg/dl)	58.79 ± 2.72	144.20 ± 15.84 ^c	43.59 ± 3.65	112.20 ± 5.67 ^c
HDL cholesterol (mg/dl)	62.85 ± 5.73	49.85 ± 4.47	61.63 ± 5.86	54.09 ± 5.84
Triglycerides (mg/dl)	90.12 ± 8.22	70.21 ± 7.36	99.67 ± 11.38	100.10 ± 5.98
Adiponectin (µg/ml)	8.65 ± 0.25	5.51 ± 0.38 ^c	9.41 ± 0.22	6.22 ± 1.16 ^c
Serum amyloid A (ng/ml)	14.78 ± 2.55	24.65 ± 3.49 ^a	24.49 ± 6.61	32.35 ± 9.43 ^d
IL-10 (pg/ml)	5.25 ± 3.65	8.37 ± 4.55 ^b	7.38 ± 2.41	9.04 ± 1.97

Total cholesterol, HDL-cholesterol, triglycerides, adiponectin, serum amyloid A, and interleukin (IL-10) were measured in plasma of corresponding animal groups at the end of 14 weeks treatment as described in Methods. Results presented are the mean ± SEM; n = 6–10.

^a *P* < 0.01, and when compared with control diet.

^b *P* < 0.05, and when compared with control diet.

^c *P* < 0.001, and when compared with control diet.

^d *P* < 0.05, when compared with wild-type mice fed the same diet.

already significantly elevated under control diet conditions and was strongly increased by the atherogenic diet.

Endothelial dysfunction measured in the aortic sinus of *Ucp2*^{-/-} mice

Immunohistochemistry analyses for endothelial nitric oxide synthase (eNOS) expression were performed on aortic sections as shown in Fig. 4A. In wild-type animals, there was a clearly detectable increase in eNOS staining of the arterial wall in response to the diet. However in *Ucp2*^{-/-} mice, even under chow-fed control conditions eNOS was already elevated compared with wild-type mice, and there was no further change in response to the atherogenic diet. Additional measures of eNOS mRNA levels were made from whole aortas collected from each group of mice. Unlike what was observed by immunohistochemistry, as shown in Fig. 4B, there was no difference in eNOS transcript levels between the two genotypes under basal conditions. However, in response to the atherogenic diet, eNOS expression rose substantially in wild-type animals (~3.1-fold), while in *Ucp2*-null mice the increase was more modest. Since most of the eNOS staining was confined to the endothelia, mRNA levels measured within the whole aorta may not be reflective of this more localized expression. In addition, there are cases in which changes in the mRNA levels differ from the expression or activity of a protein. In the case of eNOS, this discordance has been reported in some animal models of atherosclerosis (46–49), as noted further in Discussion.

The expression of the adhesion molecule vascular cell adhesion molecule-1 (VCAM-1) was then examined, since it constitutes one of the first endothelial alterations that facilitates monocyte invasion of the intimal space (50, 51). Fig. 4C shows that on the control diet there was a small insignificant difference in VCAM-1 expression between the genotypes. In response to the atherogenic diet, VCAM-1 increased in wild-type mice, while in *Ucp2*^{-/-} mice, the change in response to diet was significantly higher.

Systemic and aortic oxidative stress in *Ucp2*^{-/-} mice

Inflammation, whether localized or systemic, corresponds to a state of oxidative stress, and this certainly

pertains to atherosclerosis. Because UCP2 appears to counterbalance oxidative stress and respond to metabolically stressful challenges (22–24), as also indicated in Fig. 1, we measured a collection of oxidative stress markers in the aortae of wild-type and *Ucp2*^{-/-} mice fed either the control or atherogenic diet. In the presence of superoxide anions, nitric oxide can generate peroxynitrite, a strong oxidizing and nitrating RNS. These RNS can lead to oxidation of certain amino acid residues including the oxidation and nitration of tyrosine (52). Therefore, we determined levels of 3-nitrotyrosine in aortic sections by immunohistochemistry from wild-type and *Ucp2*^{-/-} mice. As shown in Fig. 5A, staining was undetectable in the *Ucp2*^{+/+} animals on the control diet, with a clearly visible increase in response to the atherogenic diet. However, in *Ucp2*-null mice, nitrotyrosine immunoreactivity was already apparent even under basal chow-fed conditions. Moreover, in response to the atherogenic diet, this staining was intense and significantly increased. Fig. 5B shows that for mice lacking UCP2, even when fed the control diet, there was a significant increase in steady state H₂O₂ level in the aorta. In *Ucp2*^{+/+} and *Ucp2*^{-/-} mice fed the atherogenic diet for 14 weeks, net H₂O₂ was further increased, becoming similar between the two genotypes (*P* = 0.40). Because a local redox imbalance reflects the sum failure of different antioxidant systems within the vicinity to buffer locally produced ROS, net antioxidant capacity in the aorta was measured as the production of Thiobarbituric Acid Reactive Substances by the method of Koracevic et al. (35). As shown in Fig. 5C, antioxidant capacity was similar in both genotypes under basal conditions. However, once fed the atherogenic diet wild-type animals showed a significant increase in their total aortic antioxidant capacity (~1.7-fold), while *Ucp2*^{-/-} mice failed to do so (~1.2-fold, *P* = 0.19). From these results together with those in Fig. 4A and Fig. 4C, it appears that *Ucp2*-null animals show signs of difficulty buffering an oxidative state even prior to any dietary challenge, because they show higher local ROS, higher nitrotyrosine, and higher eNOS protein. This is further supported by the effects of feeding the atherogenic diet to *Ucp2*^{-/-} mice because they show even higher nitrotyrosine staining, a smaller net increase in eNOS mRNA levels, and no increase in the total antioxidant response.

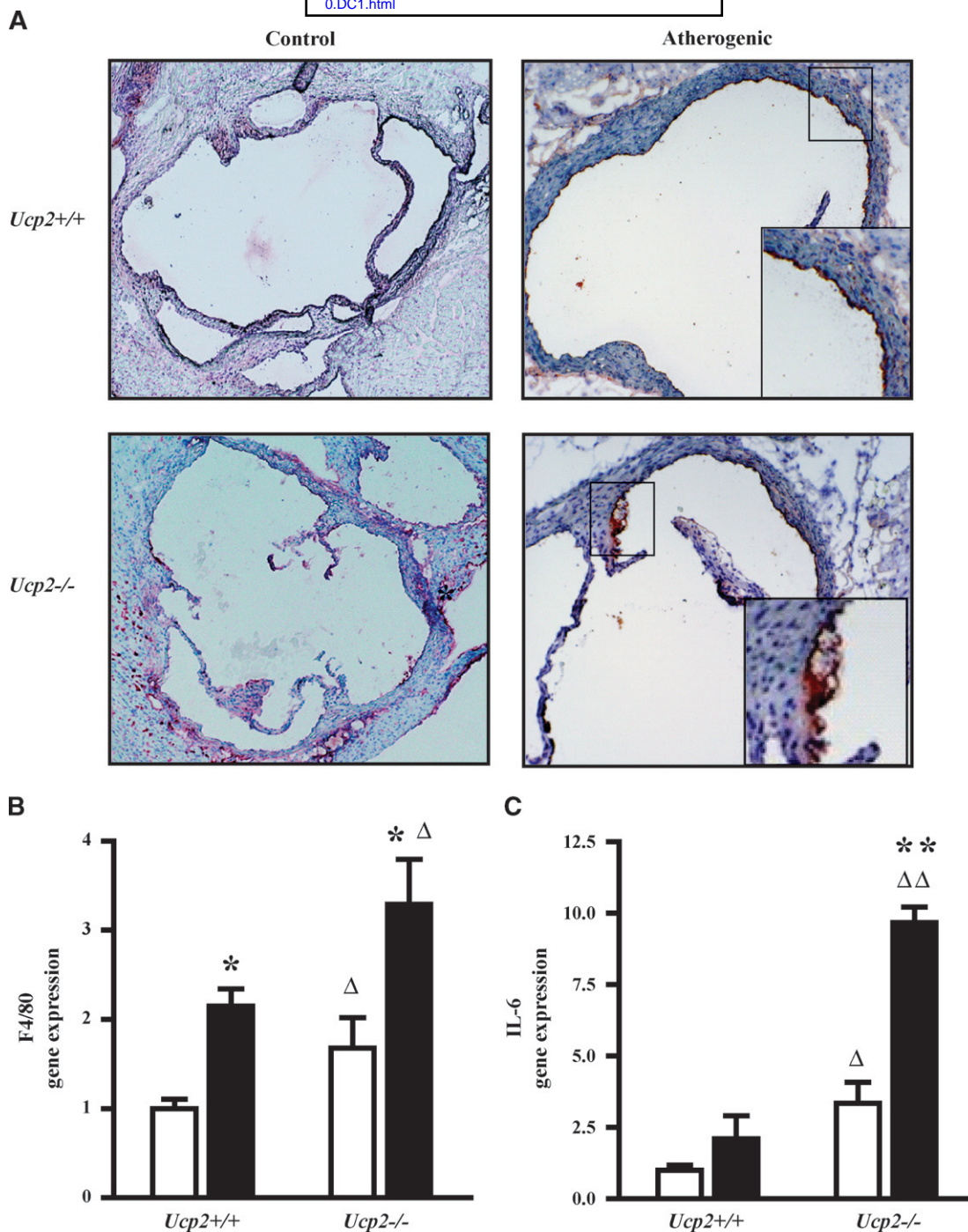


Fig. 3. Absence of UCP2 promotes macrophage infiltration in the aortic sinus in mice fed atherogenic diet and increases the inflammatory response. **A:** Representative aortic sections from *Ucp2*^{+/+} and *Ucp2*^{-/-} mice fed control or atherogenic diets for 14 weeks. Sections were immunostained for F4/80 and counterstained with hematoxylin. Original magnification $\times 100$. **B:** F4/80 and **C:** IL-6 expression levels in the aortas of *Ucp2*^{+/+} and *Ucp2*^{-/-} mice fed control (white bars) or atherogenic (black bars) diets for 14 weeks. Results are presented as mean \pm SEM; n = 6–10. * $P < 0.01$, ** $P < 0.001$, when comparing effect of diet within same genotype; Δ $P < 0.05$, $\Delta\Delta$ $P < 0.001$, when comparing effect of genotype within the same diet.

To extend these observations, we measured enzymatic activities of glutathione peroxidases (GPx), SOD, catalase in aortic extracts as described in Methods. Consistent with the local changes in the antioxidant capacity and increased H₂O₂, the absence of UCP2 was associated with a significantly reduced basal activity ($P < 0.01$) of Gpx: the

main H₂O₂ scavenging system. This is shown in **Fig. 6A**. *Ucp2*^{-/-} mice had lower GPx activity on the control diet than *Ucp2*^{+/+} mice. On the atherogenic diet, both genotypes exhibited significant increases in GPx activity, but the absolute activity level and the fold increase over basal levels were lower in *Ucp2*^{-/-} mice. SOD activity in the

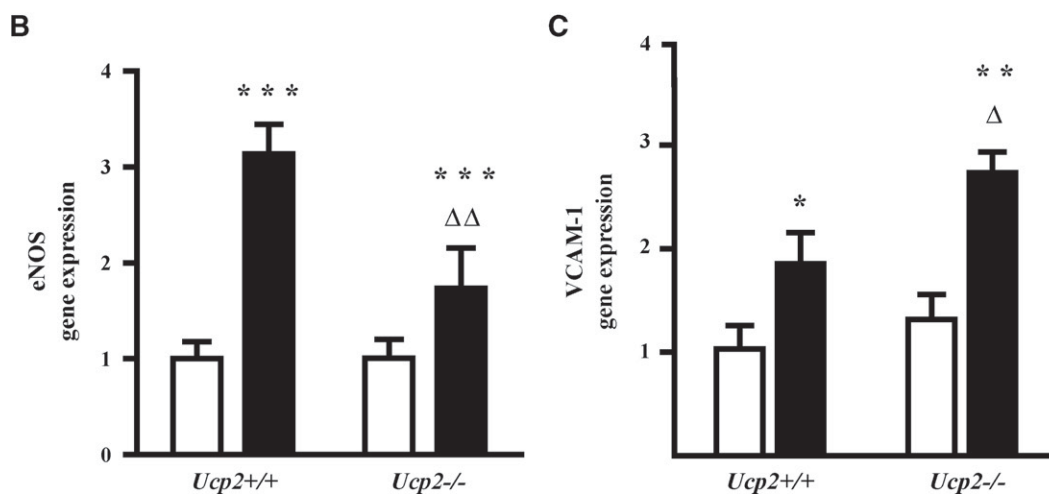
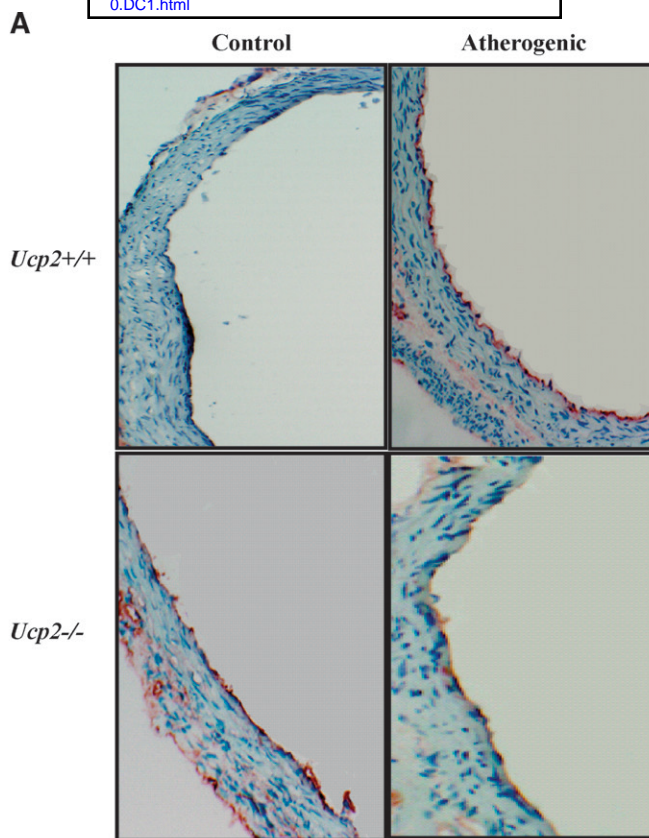


Fig. 4. Endothelial changes in the aortic sinus. Aortic sections from *Ucp2*^{+/+} and *Ucp2*^{-/-} mice fed control or atherogenic diets for 14 weeks. A: Sections were immunostained for endothelial nitric oxide synthase (eNOS) detection and counterstained with hematoxylin as described in methods. Original magnification $\times 100$. B: eNOS and C: VCAM-1 expression levels in the aortas of *Ucp2*^{+/+} and *Ucp2*^{-/-} mice fed control (white bars) or atherogenic (black bars) diets for 14 weeks. Results are presented as mean \pm SEM; n = 8–12. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, when comparing effect of diet within same genotype; Δ $P < 0.05$, $\Delta\Delta$ $P < 0.01$, when comparing effect of genotype within the same diet.

aorta of *Ucp2*^{-/-} mice was also impaired relative to wild-type mice as shown in Fig. 6B. For animals consuming the control diet, there was a small decrease in activity in *Ucp2*^{-/-} mice that did not reach statistical significance. However, SOD activity significantly increased in wild-type mice on the atherogenic diet, but in *Ucp2*-null mice there was absolutely no change. Catalase activity showed modest

insignificant increases in both genotypes in response to the atherogenic diet, and was slightly greater in *Ucp2*^{-/-} mice (Fig. 6C).

Glutathione (GSH) is a major nonenzymatic buffer and scavenger of electron deficient and otherwise chemically reactive metabolic intermediates (3). Similar to what we have observed in male *Ucp2*^{-/-} mice backcrossed into

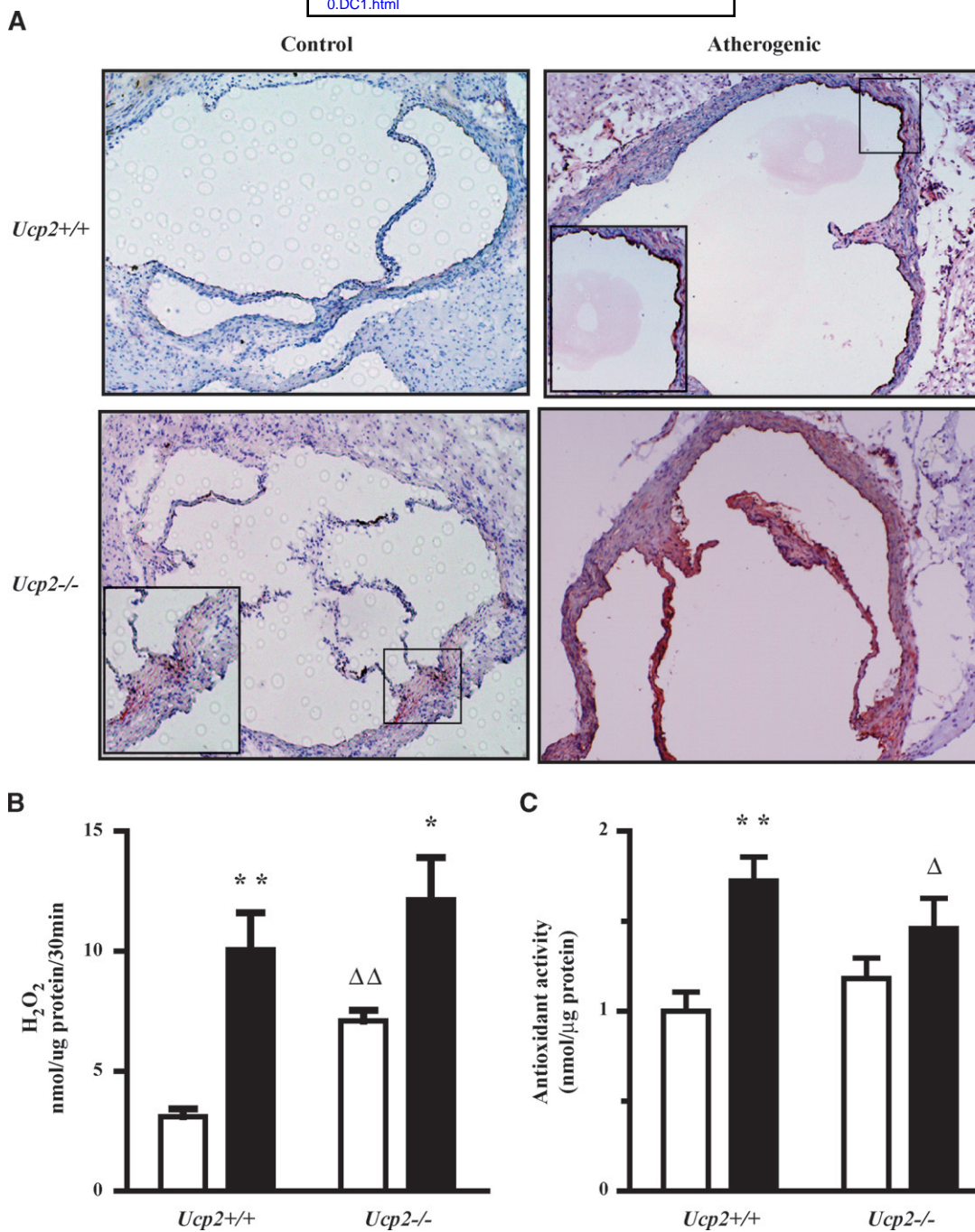


Fig. 5. Increased free radical generation in *Ucp2*^{-/-} mice. **A:** Representative aortic sections from *Ucp2*^{+/+} and *Ucp2*^{-/-} mice fed control or atherogenic diets for 14 weeks. Sections were immunostained for nitrotyrosine and counterstained with hematoxylin. Original magnification $\times 100$. **B:** Hydrogen peroxide levels and **C:** global antioxidant activity measured as Thiobarbituric Acid Reactive Substances in the aortas of *Ucp2*^{+/+} and *Ucp2*^{-/-} mice fed control (white bars) or atherogenic (black bars) diets for 14 weeks. Results are presented as mean \pm SEM; n = 6. * $P < 0.05$, ** $P < 0.01$, when comparing effect of diet within same genotype; $\Delta P < 0.05$, $\Delta\Delta P < 0.01$, when comparing effect of genotype within the same diet.

three different strain backgrounds (Pi et al., submitted), we found that, irrespective of diet, total GSH (reduced + oxidized) levels in plasma were decreased by $\sim 40\%$ in mice lacking UCP2 (Fig. 7A). In addition, there was a $\sim 99\%$ decrease in the ratio of reduced/oxidized GSH (GSH/GSSG), largely due to a dramatic increase in GSSG (Fig. 7B). These systemic changes in the glutathione system observed in the *Ucp2* null mice further support the

data regarding higher oxidative state associated with a decrease in overall antioxidant response.

DISCUSSION

Evidence in support of a role for UCP2 as a modulator of mitochondria-derived ROS continues to accrue (22, 23,

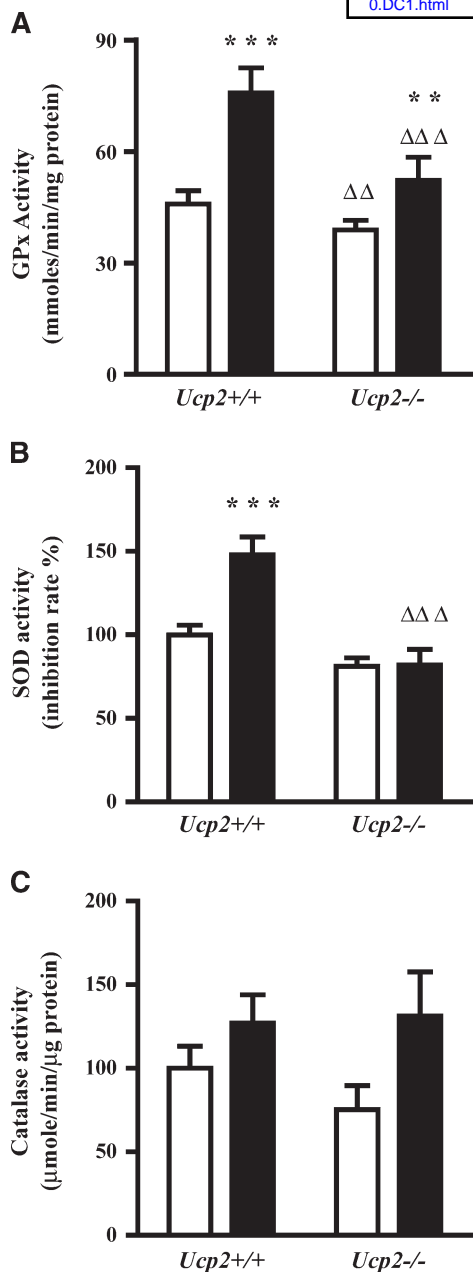


Fig. 6. Reduced antioxidant enzyme activities in the aortic sinus of *Ucp2*^{-/-} mice fed the atherogenic diet. A: Glutathione peroxidase, B: Superoxide dismutase and C: Catalase activities in the aortas of *Ucp2*^{+/+} (white bars) and *Ucp2*^{-/-} (black bars) mice fed control or atherogenic diets for 14 weeks. Results are presented as mean ± SEM; n = 6–10. ** *P* < 0.01, *** *P* < 0.001, when comparing effect of diet within same genotype; ΔΔ *P* < 0.01, ΔΔΔ *P* < 0.001, when comparing effect of genotype within the same diet.

38, 53), even as the molecular mechanism by which this occurs continues to be a focus of lively debate (as reviewed in Refs. 54, 55). Following the original observation that *Ucp2*^{-/-} mice had increased macrophage phagocytic activity and ROS generation (22), it was shown that introduction of bone marrow-derived cells from *Ucp2*^{-/-} mice into the atherosclerosis-prone LDLR^{-/-} mouse model resulted in larger lesions than recipients of wild-type cells (24). However, because UCP2 is expressed in the endothelia

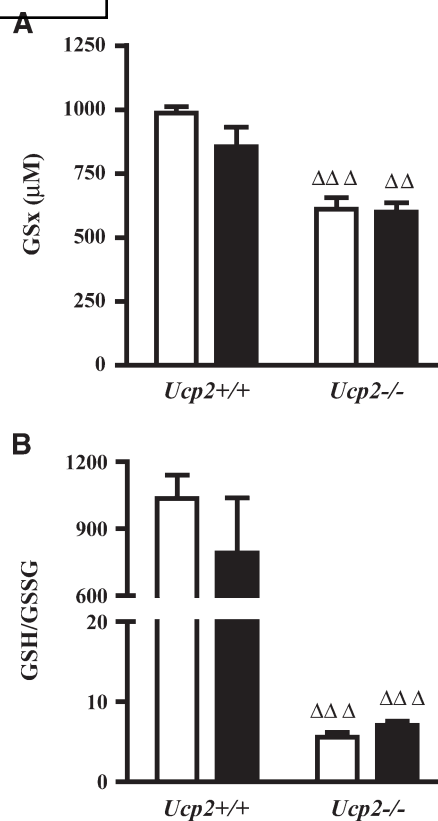


Fig. 7. Absence of UCP2 leads to glutathione depletion and a decrease in the GSH/GSSG ratio. Total glutathione levels (A) and GSH/GSSG ratio (B) measured as described in Methods in the plasma of *Ucp2*^{+/+} and *Ucp2*^{-/-} mice fed control (white bars) or atherogenic (black bars) diets for 14 weeks. Results are presented as mean ± SEM; n = 6–11; ΔΔ *P* < 0.01 and ΔΔΔ *P* < 0.001, when compared with wild-type mice fed same diet.

(25, 56), as well as the circulating macrophage population (22, 39), we wanted to evaluate if mice lacking UCP2 alone, in the absence of any other pro-atherogenic genetic manipulation, would lead to diet-induced aortic oxidative stress and consequently precipitate atherosclerotic lesion development. Among mouse strains, the B6 was shown by Paigen et al. (34) to be the most sensitive strain for developing atherosclerosis upon consumption of an atherogenic diet. It is generally recognized that these diets can provoke fatty liver in mice and can be toxic as such, and the models may not be predictive of the effect of diet per se in humans (66). Nevertheless the mouse has proven to be the foremost species for the study of experimental atherosclerosis and the influence of diet, and a valuable model because of the ability to manipulate its genome.

In the B6 *Ucp2*^{+/+} mice, the atherogenic diet triggered a significant increase in the aortic expression of UCP2, and plaque formation was minimal. In the absence of UCP2, there was a significantly increased size and number of atherosclerotic lesions. This was associated with increased systemic and local oxidative stress and a reduced antioxidant response. Together these results suggest that UCP2 is part of the protective compensatory response to this challenge in the aortic environment. Other studies have

shown that expression of UCP2 can be briskly elevated in response to a high-fat diet (21) or even during prolonged fasting when plasma fatty acids are elevated due to adipose tissue mobilization of stored triglycerides (as reviewed in Ref. 57). At this time we cannot distinguish whether the rise in UCP2 in response to the atherogenic diet is due to the lipid content per se, increased ROS derived from mitochondria via an elevated membrane potential arising from increased substrate oxidation, or ROS from another source such as lipid peroxides. Nevertheless it seems clear that UCP2 is serving a protective function against both oxidative stress and lipid accumulation in the aorta.

The adaptive increase in SOD expression and activity seen in wild-type mice was absent from *Ucp2*^{-/-} mice. Catalase activity was unaffected by the absence of UCP2, but GPx activity was also low in *Ucp2*^{-/-} mice. Interestingly superoxide is a well-established negative regulator of GPx activity (58), and thus an increase in superoxide in *Ucp2*^{-/-} mice could conceivably be a contributor to this impaired GPx activity. Since it has long been accepted that oxidative and nitrosative stresses participate in all stages of atherosclerotic lesion development (3, 59, 60), the increased protein nitration observed in the arterial wall of *Ucp2*^{-/-} mice is consistent with such increased local oxidative stress. Considering the eNOS expression in the endothelia of the two genotypes and in response to the diet, several studies using animal models of atherosclerosis have reported either unchanged or augmented expression of eNOS in atherosclerotic arteries, despite the presence of endothelial dysfunction (46–49). In addition, while inhibition of eNOS by pharmacological inhibitors or by gene knockout on the apoE^{-/-} background has been shown to promote atherogenesis (67–70), eNOS overexpression showed controversial results. Ozaki et al. (71) showed using apoE^{-/-} mice that overexpressing eNOS actually accelerated atherosclerotic lesion formation and was associated with lower NO and higher superoxide anion levels in the endothelium, sometimes referred to as an “uncoupled” state of eNOS. In *Ucp2*^{-/-} mice, a higher degree of nitrotyrosine staining as well as a higher level of eNOS protein, associated with higher levels of H₂O₂, all under basal conditions, would be consistent with a situation in which eNOS is functionally uncoupled.

Although UCP2 is expressed in endothelial cells, the greater level of UCP2 in macrophages makes it likely that a significant fraction of the increased UCP2 expression that we measured in wild-type aortas is the result of macrophage infiltration. In that respect, other markers of macrophage infiltration or inflammation, such as F4/80 and IL-6 expression, were also increased in the aortas after the atherogenic diet. However, the question of how UCP2 in endothelial cells is regulated in response to oxidative stress conditions is still unexplored. Because significant reductions in oxidative stress markers were reported in endothelial cells expressing a UCP2 transgene (31), a central antioxidant role for UCP2 in endothelia is not unlikely. This is consistent with the increased VCAM-1 expression observed in *Ucp2*^{-/-} mice. VCAM-1 levels are highly sensitive to redox status as well as NO bioavailability, suggesting

that increased superoxide and H₂O₂ accumulation are primary defects as a consequence of UCP2 deletion.

In addition to the indicators of a heightened local inflammatory response in *Ucp2*^{-/-} mice, the low levels of plasma total glutathione coupled with a significantly decreased GSH/GSSG ratio, even in the absence of the atherogenic diet challenge - are indicative of significant systemic oxidative stress. Data from other animal models are consistent with this finding, as it has also been shown that depressed glutathione synthesis precedes both oxidative stress and atherogenesis in *apoE*^{-/-} mice, which spontaneously develop very large plaques (61). Cardiac ischemia-reperfusion studies showed that tissue injury resulted from glutathione depletion and peroxide accumulation (62), while brief hypoxia before reperfusion was protective by preventing peroxide production and glutathione depletion (63). Similarly, in a patient population it was observed that there was a significant decline in cellular glutathione as well as total thiols during myocardial ischemia (64, 66). This is interesting in light of the fact that glutathione redox state recently has been identified as an independent predictor for the presence of atherosclerosis in an otherwise healthy human population (65).

In summary, our results suggest that the absence of UCP2 is associated with a collectively decreased antioxidant defense that is a favorable setting for susceptibility to atherosclerosis. Taken all together, UCP2 appears to significantly influence local superoxide and H₂O₂ production in the aorta, serving as a sensor and trigger for a protective response against lesion development. Given some linkage between the *UCP2* locus and atherosclerosis in humans (28), coupled with our findings reported here, it will be interesting in future studies of UCP2 and atherosclerosis to determine the relative contributions of UCP2 within the cell types involved in the development of the atherosclerotic plaque. ■

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