Chronic Hydroxychloroquine Improves Endothelial Dysfunction and Protects Kidney in a Mouse Model of Systemic Lupus Erythematosus

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Abstract—Hydroxychloroquine has been shown to be efficacious in the treatment of autoimmune diseases, including systemic lupus erythematosus. Hydroxychloroquine-treated lupus patients showed a lower incidence of thromboembolic disease. Endothelial dysfunction, the earliest indicator of the development of cardiovascular disease, is present in lupus. Whether hydroxychloroquine improves endothelial function in lupus is not clear. The aim of this study was to analyze the effects of hydroxychloroquine on hypertension, endothelial dysfunction, and renal injury in a female mouse model of lupus. NZBWF1 (lupus) and NZW/LacJ (control) mice were treated with hydroxychloroquine 10 mg/kg per day by oral gavage, or with tempol and apocynin in the drinking water, for 5 weeks. Hydroxychloroquine treatment did not alter lupus disease activity (assessed by plasma double-stranded DNA autoantibodies) but prevented hypertension, cardiac and renal hypertrophy, proteinuria, and renal injury in lupus mice. Aortae from lupus mice showed reduced endothelium-dependent vasodilator responses to acetylcholine and enhanced contraction to phenylephrine, which were normalized by hydroxychloroquine or antioxidant treatments. No differences among all experimental groups were found in both the relaxant responses to acetylcholine and the contractile responses to phenylephrine in rings incubated with the nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester. Vascular reactive oxygen species content and mRNA levels of nicotinamide adenine dinucleotide phosphate oxidase subunits NOX-1 and $p47^{phox}$ were increased in lupus mice and reduced by hydroxychloroquine or antioxidants. Chronic hydroxychloroquine treatment reduced hypertension, endothelial dysfunction, and organ damage in severe lupus mice, despite the persistent elevation of anti–double-stranded DNA, suggesting the involvement of new additional mechanisms to improve cardiovascular complications. (***Hypertension*****. 2014;64:330-337.)** ● **Online Data Supplement**

Key Words: acute kidney injury ■ hydroxychloroquine ■ hypertension ■ lupus erythematosus, systemic

Systemic lupus erythematosus (SLE) is a multisystemic chronic autoimmune inflammatory disorder that is associated with a high risk for the development of renal and cardiovascular disease,[1,2] which are major causes of mortality in these patients.[3] It predominantly affects young women of child-bearing age, the same population that is at lowest relative risk of atherosclerotic heart disease. In fact, women with lupus (age, 35–44 years) are >50 times as likely as healthy women without lupus to have a myocardial infarction.[4] Indeed, SLE is characterized by a high incidence of hypertension,[5–7] a well-established risk factor for the development and acceleration of atherosclerosis and ischemic heart disease. Oxidative stress and the inactivation of nitric oxide (NO) by vascular superoxide anion ($O_2^{\cdot-}$) play a critical role in the pathogenesis of endothelial dysfunction, an early event in most cardiovascular diseases, including hypertension.[8,9] Reactive oxygen species (ROS) have been considered as risk and enhancer factors for autoimmune diseases,[10] and oxidation is one of the major factors responsible for atheroma development in this context. Indeed, SLE is a disease characterized by an increased oxidative damage.[11–14] Free radical–mediated reactions are implicated in endothelial dysfunction in SLE,[15] and renal oxidative stress plays an important mechanistic role in the development of autoimmune-mediated hypertension.[16]

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Antimalarial drugs remain the first-line treatment for patients with mild SLE along with nonsteroidal anti-inflammatory drugs. The antimalarial drug hydroxychloroquine has immunomodulatory actions and has demonstrated several beneficial cardiovascular effects in patients with SLE.[17] Hydroxychloroquine has been shown to reduce serum cholesterol and low-density lipoprotein levels compared with patients treated with corticosteroids,[18] and the lipid-lowering effect of hydroxychloroquine is greater in younger patients (age, 16–39 years).[19] Patients with lupus treated with hydroxychloroquine have significantly lower mean glucose levels[20] lower fasting glucose, and markers of insulin resistance in women with SLE.[21] Hydroxychloroquine was protective against thrombovascular events,[22–24] and a negative relationship was found between the use of hydroxychloroquine and the presence of atherosclerosis.[25–27] Hydroxychloroquine has also been inversely associated with the presence of metabolic syndrome and subclinical arteriosclerosis in SLE.[28,29] Again, protective effect of hydroxychloroquine in retarding renal damage in SLE is also evident.[30] However, there is no information about the effects of hydroxychloroquine on endothelial dysfunction. Autoantibodies are involved in cardiovascular complications in SLE. However, patients treated with hydroxychloroquine showed similar plasma anti–double-stranded DNA (dsDNA) titers than untreated ones,[31] suggesting the involvement of other protective mechanisms. Hydroxychloroquine inhibited $O_2^{\cdot-}$ generation in mononuclear phagocytes stimulated by different agents,[32] but whether hydroxychloroquine is able to reduce oxidative stress in other cell types or in vivo is unknown. We hypothesized that hydroxychloroquine would reduce blood pressure and restore endothelial dysfunction. Therefore, the present study was designed to analyze the effects of hydroxychloroquine on endothelial dysfunction, oxidative stress, and renal injury in a mouse model of SLE (female NZBWF1 mice) and whether an in vivo antioxidant effect was involved.

Materials and Methods

The investigation conforms to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and our Institutional Guidelines for the ethical care of animals. Thirty-week-old female NZBWF1 (SLE) and NZW/LacJ (control) mice obtained from Jackson Laboratories (Bar Harbor, ME) were randomly assigned to receive by oral gavage hydroxychloroquine (10 mg/kg per day) or vehicle (1 mL of 1% methylcellulose) or a combination of the antioxidants tempol (2.0 mmol/L) and apocynin (1.5 mmol/L) in the drinking water[16] for 5 weeks. Mice were randomly divided into 5 groups: control untreated (Ctrl-Veh), control treated with hydroxychloroquine, SLE untreated (SLE-Veh), SLE treated with hydroxychloroquine (SLE-HCQ), and SLE treated with tempol and apocynin. Systolic blood pressure (SBP) and heart rate were measured by tail-cuff plethysmography and by intra-arterial register. At the end of the experiment, plasma anti-dsDNA antibodies, plasma malonyldialdehyde, physical characteristics, cardiac and renal weight indices, and proteinuria were measured. Histopathologic evaluation of kidneys was also performed in sections stained with hematoxylin–eosin, Periodic acid–Schiff, and Masson trichrome. Descending thoracic aortic rings were mounted in a wire myograph for isometric tension measurement. ROS levels were estimated from the ratio of ethidium/4,6-diamidino-2-phenylindole dichlorohydrate (DAPI) fluorescence in sections of unfixed thoracic aortic rings incubated with dihydroethidium and counterstained with the nuclear stain DAPI. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity was measured by the lucigenin-enhanced chemiluminescence assay. Flow cytometry was used to measure B (positive to anti-B220$^+$) and T cells (positive to anti-CD4$^+$, total T; anti-CD4$^+$ FOXP3$^+$, regulatory T cells; anti-CD4$^+$ IL17$^+$, Th17; and anti-CD4$^+$ IFNγ^+, Th1) from spleens. Protein and mRNA expression were measured by Western blotting analysis and reverse transcriptase-polymerase chain reaction analysis, respectively. An expanded Methods section is available in the online-only Data Supplement.

Results

Effects of Hydroxychloroquine on Blood Pressure, Cardiac and Renal Hypertrophy, Spleen Lymphocytes, and Plasma Determinations

The time course of tail SBP is shown in Figure 1A, and the final SBP and heart rate measured by direct recordings are shown in Figure 1B. Initial SBP was significantly higher in SLE mice compared with controls. No significant changes in SBP were induced by 5 weeks of vehicle treatment either in SLE or in control mice. Both hydroxychloroquine and antioxidant treatments reduced SBP in SLE mice (Figure 1A and 1B), being without effects on heart rate (Figure 1B). Heart weight/tibia length and kidney weight/tibia length indices were higher in SLE-Veh (0.076±0.003 and 0.100±0.006 g/cm, respectively) than in Ctrl-Veh mice (0.067±0.002 and 0.082±0.002 g/cm, respectively). Hydroxychloroquine treatment reduced significantly (P<0.05) both cardiac and renal indices only in SLE mice (0.066±0.002 and 0.085±0.002 g/cm, respectively), whereas

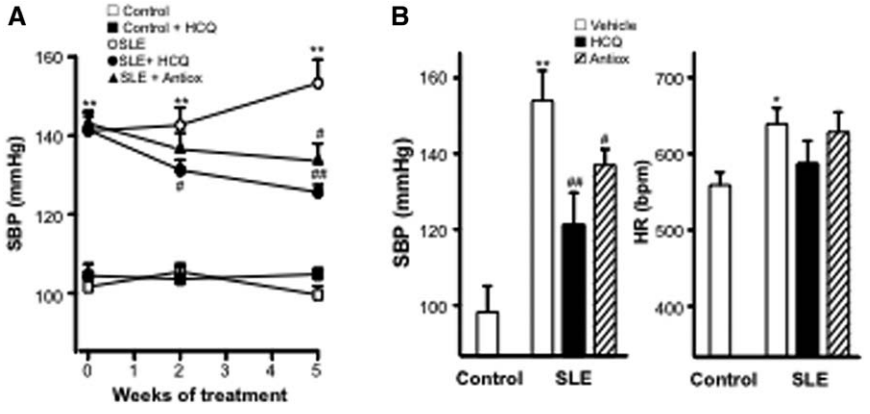

Figure 1. Effects of chronic hydroxychloroquine (HCQ) and antioxidant (Antiox; tempol+apocynin) treatment on systolic blood pressure (SBP) and heart rate (HR) measured by tail-cuff plethysmography (**A**) or by direct register in left carotid artery (**B**) in control and systemic lupus erythematosus (SLE) mice. Experimental groups: Control (n=17), control treated with HCQ (Control-HCQ; 10 mg/kg per day; n=10), SLE untreated (n=11), SLE treated with hydroxychloroquine (SLE-HCQ; n=13), SLE treated with Antiox (SLE-Antiox; n=7). Values are expressed as mean±SEM (n=7–17). **P<0.01 and *P<0.05 vs control group. ##P<0.01 and #P<0.05 vs SLE group.

the antioxidant mixture was without effect (0.076±0.004 and 0.111±0.007 g/cm, respectively).

Total plasma anti-dsDNA (IgG) antibodies were significantly (P<0.01) greater in SLE mice (107±8 ng/mL) compared with control mice (56±8 ng/mL) as reported previously. As expected, treatment with hydroxychloroquine did not modify the levels of anti-dsDNA in SLE (101±10 ng/mL) and control animals (64±16 ng/mL). Similarly, antioxidant treatment did not alter this parameter in SLE mice (98±13 ng/mL). No significant differences in plasma glucose were observed among groups (Ctrl-Veh, 100±3 mg/dL; control treated with hydroxychloroquine, 99±3 mg/dL; SLE-Veh, 96±8 mg/dL; SLE-HCQ, 95±7 mg/dL; and SLE treated with tempol and apocynin, 97±6 mg/dL).

To determine the immunomodulatory actions of hydroxychloroquine, we measured the levels of B and T cells in spleens from all experimental groups (Figure S1 in the online-only Data Supplement). No significant changes were observed among groups in both total T (Figure S1A) and B (Figure S1B) cells. However, the percentage of regulatory T cells and Th17 were increased in splenocytes from SLE mice. Hydroxychloroquine treatment decreased the percentage of both T-cell types, the antioxidant mixture being without effect. There were no differences in the percentages of Th1 cells in the spleens from all experimental groups (Figure S1C).

To test whether systemic oxidative stress is modified by treatments, we measured plasma malonyldialdehyde. This marker of lipid peroxidation was increased in plasma from SLE mice as compared with control group, and it was decreased by both hydroxychloroquine and antioxidant treatments (Figure S2).

Effects of Hydroxychloroquine on Renal Injury

Urinary Protein Excretion

Urinary protein excretion was increased in SLE mice compared with controls and significantly reduced with hydroxychloroquine treatment (Figure 2A), the combination of antioxidants being without effect.

Morphological Results

The comparative study of renal injury in different mice groups is shown in Table S2, and representative micrographs are shown in Figure 2B. SLE-Veh group showed diffuse and segmental endocapillary and mesangial hypercellularity, matrix expansion with hyalinosis, capillary wall thickening with wire-loop lesions, hyaline thrombi in lumen of tuft capillary, early segmental capillary necrosis, and moderate extracapillary proliferation (crescent in 62% of glomeruli). Scattered glomerular cyst could also be observed. Cortical tubules showed numerous hyaline casts and moderate/severe clusters of renal papillae and tubulointerstitial chronic inflammatory infiltrate. The percentage of glomeruli exhibiting a severe mesangial sclerosis area was 54.5% in SLE-Veh mice; in contrast, only a moderate mesangial sclerosis was observed in 8.3% of SLE-HCQ mice. Mesangial hyalinization, extracapillary proliferation (crescent), wire loop, fibrinoid necrosis, casts and inflammatory infiltrate, and tubular casts were greater in SLE-Veh mice compared with control mice. SLE-HCQ mice had only mild glomerular lesions (mesangial hyalinization and fibrinoid necrosis) and almost no extracapillary crescents and tubulointerstitial lesions. Thus, SLE mice treated with hydroxychloroquine showed a significant reduction of hyalinization (P=0.003), fibrinoid necrosis (P=0.01), and crescents (P=0.047, Mann–Whitney U test). In contrast, antioxidant treatment did not reduce the inflammatory infiltrate, mesangial hyalinization, or fibrinoid necrosis.

Effects of Hydroxychloroquine on Vascular Reactivity

Aorta from SLE mice showed strongly reduced endothelium-dependent vasodilator responses to acetylcholine

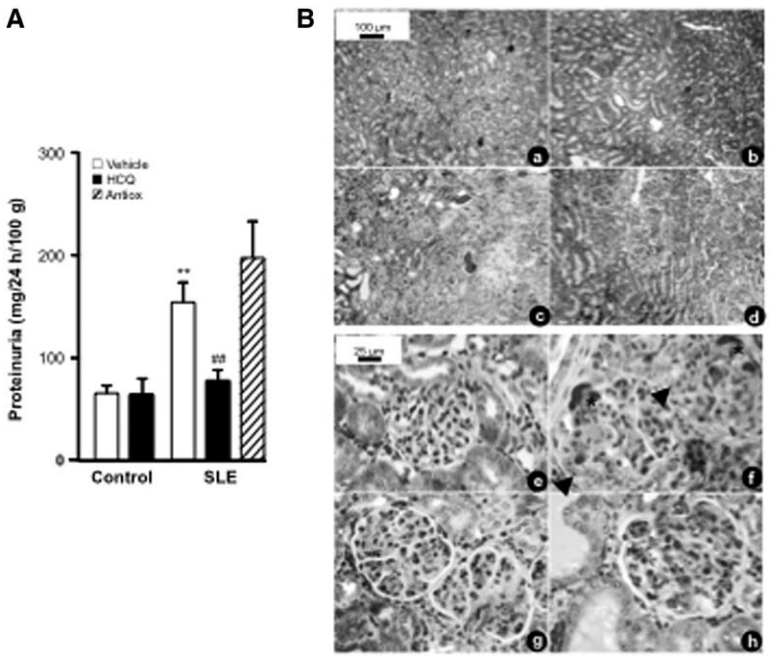

Figure 2. Effects of chronic hydroxychloroquine (HCQ) and antioxidant (Antiox; tempol+apocynin) treatment on proteinuria (**A**) and morphological renal cortex features (**B**) systemic lupus erythematosus (SLE) mice. **a**, Normal renal parenchyma in control group. **b**, Absence of glomerular or tubulointerstitial lesions in control HCQ-treated mice. **c**, SLE group with diffuse mesangial hyalinosis (green color), fucsinophil deposits, and crescents in glomeruli and hyaline casts in tubules. **d**, Persistence of mild/moderate mesangial hyalinosis in the absence of other parenchimatous injuries in SLE treated with hydroxychloroquine (SLE-HCQ) group. **e**, Detail of normal renal glomerulus in control group. **f**, Detail of mesangial hyalinosis, fucsinophil immunocomplex deposits (asterisk), and crescents (arrowhead) in SLE mice. **g**, Detail of mesangial hyalinosis in SLE-HCQ mice. **h**, Persistence of small fucsinophil immunocomplex deposits (asterisk) in SLE treated with Antiox (SLE-Antiox) mice (Masson trichrome; **a** and **b,** magnification ×2; **c** and **d**, magnification ×4; **e**–**h**, magnification ×40). Experimental groups: Control (n=17), control treated with HCQ (Control-HCQ; 10 mg/kg per day; n=10), SLE untreated (n=11), SLE-HCQ (n=13), SLE-Antiox (n=7). Values are expressed as mean±SEM (n=7–17). **P<0.01 as compared with the control group. ##P<0.01 as compared with the SLE-vehicle group.

(maximal effect, 33.3±6.5% versus 57.5±6.1% in the control group; $P<0.05$). The treatment of SLE mice with hydroxychloroquine or antioxidants showed an increase in the acetylcholine-induced vasodilation as compared with vehicle-treated SLE mice (maximal effect, 56.0±5.6%; $P<0.05$ and 58.0±7.9%; $P<0.05$, respectively; Figure 3A). These relaxant responses were suppressed by incubation for 30 minutes with the NO synthase (NOS) inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) in all experimental groups (Figure 3B). Incubation with tempol for 30 minutes increased the response to acetylcholine in rings from SLE-Veh mice (Figure 3C). We also found increased contractile response to phenylephrine in endothelium-intact aortic rings from SLE mice (maximal effect, 9.7±0.8 mN) as compared with control mice (maximal effect, 6.5±0.4 mN; $P<0.01$), control treated with hydroxychloroquine (maximal effect, 6.8±0.6 mN; $P<0.01$; Figure 3D). No significant differences in this contractile response to phenylephrine between SLE-Veh and Ctrl-Veh groups were found when the rings were incubated in the presence of L-NAME (Figure 3E). Hydroxychloroquine or antioxidant treatments suppressed the hyper-responsiveness to phenylephrine in intact rings (maximal effect, 5.9±0.7 mN; $P<0.01$ and 5.5±1.2 mN; $P<0.01$, respectively; Figure 3D). This inhibitory effect was abolished by L-NAME, suggesting a higher NO formation in these vessels compared with those from SLE mice. No differences were observed among all experimental groups in the endothelium-independent relaxant response to sodium nitroprusside (Figure 3F).

Effects of Hydroxychloroquine on Vascular ROS Levels and NADPH Oxidase Activity

To characterize and localize ROS levels within the vascular wall, ethidium red fluorescence was analyzed in sections of aorta incubated with dihydroethidium. Positive red nuclei could be observed in adventitial, medial, and endothelial cells from sections of aorta incubated with dihydroethidium (Figure 4A). In preliminary experiments, dihydroethidium fluorescence was almost abolished by tiron ($O_2^{\cdot-}$ scavenger) and pegylated superoxide dismutase. The inhibitory effect of both $O_2^{\cdot-}$ scavengers suggests that the primary source of oxidant stress is likely to be $O_2^{\cdot-}$.[33] Rings from SLE-Veh group showed marked increased staining in adventitial, medial, and endothelial cells as compared with Ctrl-Veh group, which was significantly reduced by both hydroxychloroquine and antioxidant treatment (Figure 4A and 4B).

NADPH oxidase activity was increased in aortic rings from SLE-Veh mice as compared with control mice (Figure 4C). Chronic treatment with hydroxychloroquine or the antioxidant mixture significantly reduced the NADPH oxidase activity in SLE mice.

Effects of Hydroxychloroquine on Vascular Gene Expression and on NO Activity

Endothelial NOS (eNOS) mRNA expression was similar among all experimental groups (Figure 5A). Significant mRNA overexpression of NADPH oxidase subunits NOX-1 (Figure 5B) and $p47^{phox}$ (Figure 5C), without change of $p22^{phox}$ (Figure 5D), were observed in aortic tissues from SLE-Veh as compared with control mice. Hydroxychloroquine and antioxidant treatment reduced gene expression of both subunits in SLE but not in control mice.

The phosphorylation of vasodilator-stimulated phosphoprotein was measured and used as an estimate of NO levels.[34] Reduced vasodilator-stimulated phosphoprotein phosphorylation was found in aorta from SLE-Veh group as compared with the control group. Both hydroxychloroquine and antioxidants increased the levels of this biochemical marker for monitoring NO in SLE mice (Figure 5E).

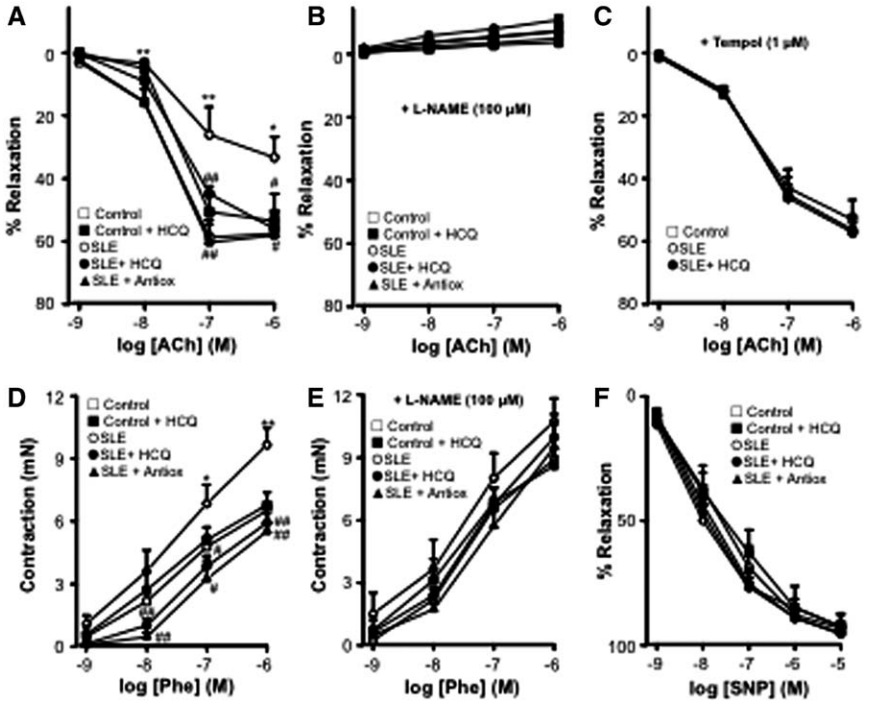

Figure 3. Effects of chronic hydroxychloroquine (HCQ) and antioxidant (Antiox; tempol+apocynin) treatment on endothelial function. Endothelium-dependent vasodilator responses to acetylcholine (ACh) in intact aortic rings precontracted with phenylephrine (Phe) in the absence (**A**) or in the presence (**B**) of N^G-nitro-L-arginine methyl ester (L-NAME), or tempol (**C**). Vasoconstriction induced by Phe in intact aortic rings in the absence (**D**) or in the presence (**E**) of L-NAME. Endothelium-independent vasodilator responses to sodium nitroprusside (SNP) in arteries previously contracted by 10^{-6} mol/L Phe (**F**). Experimental groups: Control (n=17), control treated with HCQ (Control-HCQ; 10 mg/kg per day; n=10), systemic lupus erythematosus untreated (SLE; n=11), SLE treated with HCQ (SLE-HCQ; n=13), SLE-Antiox (n=7). Values are expressed as mean±SEM (n=7–17). $^{**}P<0.01$ and $^{*}P<0.05$ vs control group. $\#\#P<0.01$ and $\#P<0.05$ vs SLE group.

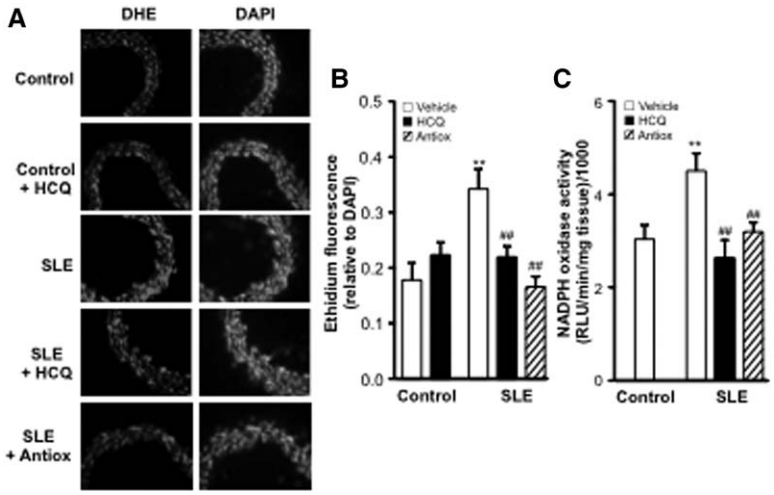

Figure 4. Effects of chronic hydroxychloroquine (HCQ) and antioxidant (Antiox; tempol+apocynin) treatment on in situ localization of O_2^- and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity in aortic rings. **A**, **Left**, Arteries incubated in the presence of dihydroethidium (DHE) which produces a red fluorescence when oxidized to ethidium by O_2^-. **Right**, Blue fluorescence of the nuclear stain 4,6-diamidino-2-phenylindole dichlorohydrate (DAPI; magnification ×400). **B**, Averaged values, mean±SEM (n=5–6), of red ethidium fluorescence normalized to blue DAPI. **C**, NADPH oxidase activity measured by chemiluminescence with lucigenin. Experimental groups: Control (n=17), control treated with HCQ (Control-HCQ; 10 mg/kg per day; n=10), systemic lupus erythematosus untreated (SLE; n=11), SLE treated with HCQ (SLE-HCQ; n=13), SLE treated with Antiox (SLE-Antiox; n=7). Values are expressed as mean±SEM (n=7–17). ***P*<0.01 vs control group. ##*P*<0.01 vs SLE group.

Discussion

In the present study, we investigated whether the antimalarial drug hydroxychloroquine therapy reduces cardiovascular complications in a model of autoimmune disease with hypertension. The major new findings of the present study are as follows: (1) chronic hydroxychloroquine reduced the elevated SBP, (2) it reduced heart and kidney hypertrophy, (3) it restored endothelial function in SLE, and (4) these protective effects seem to be related to decreased ROS production as a result of NADPH oxidase subunit downregulation and increased NO bioavailability. Likewise, the effects of hydroxychloroquine on endothelial function were mimicked by a mixture of antioxidants. This study also confirms that hydroxychloroquine treatment did not alter SLE disease activity (assessed by plasma dsDNA autoantibodies) in the mice model and prevented the morphological lesions and proteinuria found in SLE mice.

SLE is associated with a high prevalence of hypertension.[6,35] This increase in blood pressure does not seem to be dependent on glomerulonephritis that is also highly prevalent in individuals with SLE.[36–38] In our experiment, hydroxychloroquine treatment of SLE mice partially reduced the glomerular lesions and almost fully prevented the extracapillary crescents and tubulointerstitial lesions and proteinuria and reduced SBP. A mixture of antioxidants containing tempol and apocynin was used for comparative purposes. This antioxidant treatment reduced SBP as previously described but was unable to prevent the renal lesions, which contrasts with data previously found in this same model of SLE.[16] Thus, our results are in agreement with the hypothesis that SLE is a risk factor for hypertension and can be controlled independently of nephritis.[39]

Our results are also consistent with previous evidences showing that hydroxychloroquine did not reduce plasma anti-DNA

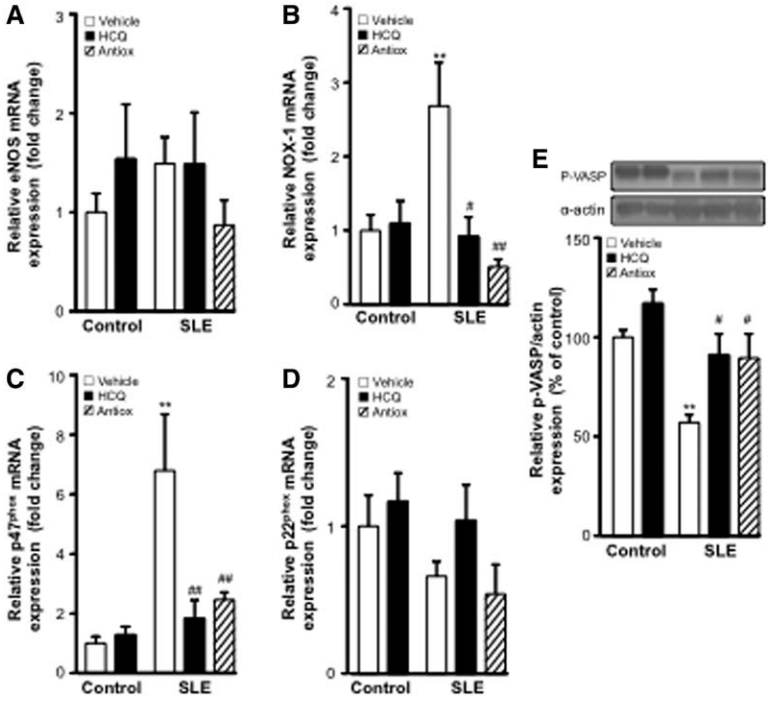

Figure 5. mRNA expression of endothelial nitric oxide synthase (eNOS; **A**), NOX-1 (**B**), p47^{phox} (**C**), p22^{phox} (**D**), and vasodilator-stimulated phosphoprotein (VASP) phosphorylation (**E**) in aortic rings from all experimental groups. Data are presented as a ratio of arbitrary units of mRNA ($2^{-\Delta\Delta Ct}$) or phospho-VASP (p-VASP)/actin ratio compared with control. Values are expressed as mean±SEM (n=7–17). Experimental groups: Control (n=17), control treated with HCQ (Control-HCQ; 10 mg/kg per day; n=10), systemic lupus erythematosus untreated (SLE; n=11), SLE treated with HCQ (SLE-HCQ; n=13), SLE treated with Antiox (SLE-Antiox; n=7). ***P*<0.01 as compared with the control group. #*P*<0.05 and ##*P*<0.01 as compared with the SLE group.

titers[31] but prevented renal damage in patients with SLE.[30] This lack of effect on autoantibody generation correlated with the absence of change in production of B cells in spleens observed after hydroxychloroquine treatment. However, hydroxychloroquine reduced the percentage of both Treg and Th17 cells which is increased in SLE mice. This effect might be related with the inhibitory effect on lymphocyte proliferation induced by this agent.[40,41] Infiltration of T cells into the kidney is a typical feature of human and experimental lupus nephritis that contributes to renal tissue injury.[42,43] Thus, reducing Th17 cells by hydroxychloroquine treatment might be the main mechanism involved in the protective effects of hydroxychloroquine in lupus nephritis. Likewise, the antioxidant mixture, which failed to affect the T-cell profile, was also without effect on the renal injury and proteinuria in SLE mice. This suggests that mechanisms besides the antioxidant effect should be involved in the renal protective effects of hydroxychloroquine in SLE mice. One of the known mechanisms of action of hydroxychloroquine in lupus is to reduce the acidity inside cells, which would lead to decreased antigen presentation. This would result in reduced toll-like receptor 7 and 9 signaling which would decrease inflammation and ROS production. This may also explain some of the differences between hydroxychloroquine effects and tempol/apocynin effects.

Hypertension is often associated with impaired endothelial function, but whether this is causative in the progression of hypertension is difficult to prove. Numerous studies suggest that the endothelium is prominently affected during SLE, as demonstrated by the high risk for the development of atherosclerosis.[44,45] In fact, macrophage activation and intima infiltration, and in turn atherogenesis, are often preceded by endothelial damage or dysfunction. Endothelial cell dysfunction represents the earliest indication of the development of cardiovascular disease and is also a principal element of SLE.[46] In large vessels, flow-mediated dilatation is impaired in patients with SLE.[47–49] However, the cutaneous microvascular reactivity to local heat or iontophoretically administered acetylcholine did not differ between patients with SLE and matched controls.[50] However, in NZBWF1 mice, which have uniform genetics and environmental conditions, we found impaired aortic endothelium-dependent relaxation response to acetylcholine. Interestingly, Ryan and McLemore[51] found that the impaired response to acetylcholine begins before the development of proteinuria and increased blood pressure, suggesting that early changes in vessel function may contribute to the development of hypertension during SLE. Our findings extend the role of endothelial dysfunction in SLE and hypertension, because hydroxychloroquine and antioxidants were able to restore acetylcholine relaxation and the increased SBP. Moreover, in SLE-Veh mice, we found increased heart weight/tibia length as compared with control mice. Treatment with hydroxychloroquine in SLE mice was accompanied by a significant reduction in this parameter. However, the antioxidant treatment, which also improved endothelial dysfunction and slowly reduced SBP, did not reduce cardiac hypertrophy. This lack of beneficial effects might be related with the different time course of antihypertensive effects of both treatments, showing that longer and intense SBP reductions are required to reduce hypertrophy.

Inflammatory responses in the endothelium induced by circulating autoantibodies and other inflammatory mediators are known to contribute to the pathogenesis of endothelial dysfunction, and numerous studies implicate cytokines in the progression of SLE.[52] However, the molecular mechanisms involved in endothelial dysfunction in SLE mice have never been analyzed. NO secretion is required for normal endothelium-dependent vasodilatation. Patients with SLE display a defect in the function of eNOS in endothelial cells.[49] We found that endothelium-dependent relaxations induced by acetylcholine were abolished by eNOS inhibition with L-NAME. Moreover, increased contractile responses to phenylephrine were observed in aorta from SLE-Veh group as compared with control mice, which were suppressed by L-NAME, suggesting a defect in NO pathway in SLE mice. However, changes in both acetylcholine-mediated relaxation and phenylephrine-induced contraction in SLE mice do not seem to be related to changes in eNOS mRNA expression, suggesting no changes in NO production under basal or stimulated conditions. However, a detailed analysis of eNOS protein levels, phosphorylation state, and NO release will be required to definitively answer this question.[51] Interestingly, the improvement in both acetylcholine relaxation and phenylephrine contractions induced by hydroxychloroquine in SLE was suppressed by L-NAME, suggesting that hydroxychloroquine improved the eNOS pathway in aortic tissue. This protective effect seems to be unrelated to changes in eNOS mRNA expression and also to changes in the sensitivity to the NO-cGMP pathway because the vasodilator response to the NO donor, nitroprusside, was unaffected by hydroxychloroquine treatment.

A key mechanism of endothelial dysfunction involves the vascular production of ROS, particularly $O_2^{\cdot-}$, which reacts rapidly with and inactivates NO.[53] We found for the first time that ROS levels are increased in aorta from SLE-Veh and that hydroxychloroquine reduced ROS content, which would be involved in its protective effects on endothelial function. In fact, the impaired endothelium-dependent relaxation found in aorta from SLE mice was restored by both acute incubation with the superoxide dismutase mimetic tempol and by chronic antioxidant treatment. The NADPH oxidase, a multienzymatic complex formed by $gp91^{phox}$ or its vascular homologous NOX-1 and NOX-4, rac, $p22^{phox}$, $p47^{phox}$, and $p67^{phox}$, is considered the major source of $O_2^{\cdot-}$ in the vascular wall. We found a marked increase in aortic NADPH oxidase activity in SLE mice, accompanied with an increase in mRNA of $p47^{phox}$ and NOX-1, being without effect on $p22^{phox}$. Hydroxychloroquine and antioxidant treatments inhibited the upregulation of these subunits in SLE mice. A previous in vitro study reported that hydroxychloroquine inhibited $O_2^{\cdot-}$ production on human polymorphonuclear neutrophils stimulated by opsonized zymosan, phorbol myristate acetate, or fluoride.[32] Herein, we show that hydroxychloroquine not only inhibits the activity, but also the expression of NADPH oxidase, the main vascular source of $O_2^{\cdot-}$. Moreover, vasodilator-stimulated phosphoprotein phosphorylation was reduced in SLE mice, and it was increased after treatment with hydroxychloroquine or antioxidants. Taken into account that endothelium-derived NO is a major contributor to vasodilator-stimulated phosphoprotein phosphorylation in

vascular tissue,[39] our data suggest increased NO bioavailability after both treatments. Taken together, the results suggest that the reduction of $O_2^{\cdot-}$ derived from NADPH oxidase in the vascular wall and the subsequent prevention of NO inactivation constitute the main mechanisms involved in its protective effects on endothelial function. However, other changes in antioxidant defense system could be involved in the reduced vascular ROS level found in SLE-HCQ group. The potential contribution of these mechanisms in hydroxychloroquine treatment has not been established here.

In conclusion, our study demonstrates that in SLE mice, chronic hydroxychloroquine treatment improves endothelium-dependent relaxation, essentially by preserving the NO-mediated component, and reduces SBP. This protective effect may be attributable to a decrease in the vascular oxidative stress by normalizing the expression of NADPH oxidase subunits.

Perspectives

We suggest that these vascular effects, together with the antiaggregant actions, the improvement of the dyslipidemia, and the glucose intolerance induced by hydroxychloroquine, are involved in the lower incidence of thromboembolic disease found in hydroxychloroquine-treated SLE patients. Moreover, these results reinforce the notion that hydroxychloroquine should be used not only in patients with mild SLE disease, but also in those with major organ involvement.

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Disclosures

None.

References

1. Frostegård J. Systemic lupus erythematosus and cardiovascular disease. *Lupus*. 2008;17:364–367.
2. Hahn BH, McMahon M. Atherosclerosis and systemic lupus erythematosus: the role of altered lipids and of autoantibodies. *Lupus*. 2008;17:368–370.
3. Skamra C, Ramsey-Goldman R. Management of cardiovascular complications in systemic lupus erythematosus. *Int J Clin Rheumtol*. 2010;5:75–100.
4. Manzi S, Meilahn EN, Rairie JE, Conte CG, Medsger TA Jr, Jansen-McWilliams L, D'Agostino RB, Kuller LH. Age-specific incidence rates of myocardial infarction and angina in women with systemic lupus erythematosus: comparison with the Framingham Study. *Am J Epidemiol*. 1997;145:408–415.
5. Petri M. Hydroxychloroquine: past, present, future. *Lupus*. 1998;7:65–67.
6. Al-Herz A, Ensworth S, Shojania K, Esdaile JM. Cardiovascular risk factor screening in systemic lupus erythematosus. *J Rheumatol*. 2003;30:493–496.
7. Urowitz MB, Gladman D, Ibañez D, et al. Clinical manifestations and coronary artery disease risk factors at diagnosis of systemic lupus erythematosus: data from an international inception cohort. *Lupus*. 2007;16:731–735.
8. Griendling KK, Alexander RW. Oxidative stress and cardiovascular disease. *Circulation*. 1997;96:3264–3265.
9. Landmesser U, Harrison DG. Oxidative stress and vascular damage in hypertension. *Coron Artery Dis*. 2001;12:455–461.
10. Mansour RB, Lassoued S, Gargouri B, El Gaïd A, Attia H, Fakhfakh F. Increased levels of autoantibodies against catalase and superoxide dismutase associated with oxidative stress in patients with rheumatoid arthritis and systemic lupus erythematosus. *Scand J Rheumatol*. 2008;37:103–108.
11. Kang TY, El-Sohemy A, Comelis MC, Eny KM, Bae SC. Glutathione S-transferase genotype and risk of systemic lupus erythematosus in Koreans. *Lupus*. 2005;14:381–384.
12. Sheikh Z, Ahmad R, Sheikh N, Ali R. Enhanced recognition of reactive oxygen species damaged human serum albumin by circulating systemic lupus erythematosus autoantibodies. *Autoimmunity*. 2007;40:512–520.
13. Ben Mansour R, Lassoued S, Elgaied A, Haddouk S, Marzouk S, Bahloul Z, Masmoudi H, Attia H, Aïfa MS, Fakhfakh F. Enhanced reactivity to malondialdehyde-modified proteins by systemic lupus erythematosus autoantibodies. *Scand J Rheumatol*. 2010;39:247–253.
14. Hassan SZ, Gheita TA, Kenawy SA, Fahim AT, El-Sorougy IM, Abdou MS. Oxidative stress in systemic lupus erythematosus and rheumatoid arthritis patients: relationship to disease manifestations and activity. *Int J Rheum Dis*. 2011;14:325–331.
15. Kahlenberg JM, Kaplan MJ. The interplay of inflammation and cardiovascular disease in systemic lupus erythematosus. *Arthritis Res Ther*. 2011;13:203.
16. Mathis KW, Venegas-Pont M, Masterson CW, Stewart NJ, Wasson KL, Ryan MJ. Oxidative stress promotes hypertension and albuminuria during the autoimmune disease systemic lupus erythematosus. *Hypertension*. 2012;59:673–679.
17. Ruiz-Irastorza G, Ramos-Casals M, Brito-Zeron P, Khamashta MA. Clinical efficacy and side effects of antimalarials in systemic lupus erythematosus: a systematic review. *Ann Rheum Dis*. 2010;69:20–28.
18. Wallace DJ, Linker-Israeli M, Hyun S, Klinenberg JR, Stecher V. The effect of hydroxychloroquine therapy on serum levels of immunoregulatory molecules in patients with systemic lupus erythematosus. *J Rheumatol*. 1994;21:375–376.
19. Wallace DJ, Metzger AL, Stecher VJ, Turnbull BA, Kern PA. Cholesterol-lowering effect of hydroxychloroquine in patients with rheumatic disease: reversal of deleterious effects of steroids on lipids. *Am J Med*. 1990;89:322–326.
20. Petri M. Hydroxychloroquine use in the Baltimore Lupus Cohort: effects on lipids, glucose and thrombosis. *Lupus*. 1996;5(suppl 1):S16–S22.
21. Penn SK, Kao AH, Schott LL, Elliott JR, Toledo FG, Kuller L, Manzi S, Wasko MC. Hydroxychloroquine and glycemia in women with rheumatoid arthritis and systemic lupus erythematosus. *J Rheumatol*. 2010;37:1136–1142.
22. Wallace DJ. Does hydroxychloroquine sulfate prevent clot formation in systemic lupus erythematosus? *Arthritis Rheum*. 1987;30:1435–1436.
23. Pierangeli SS, Harris EN. *In vivo* models of thrombosis for the antiphospholipid syndrome. *Lupus*. 1996;5:451–455.
24. Becker-Merok A, Nossent J. Prevalence, predictors and outcome of vascular damage in systemic lupus erythematosus. *Lupus*. 2009;18:508–515.
25. Petri M, Perez-Gutthann S, Spence D, Hochberg MC. Risk factors for coronary artery disease in patients with systemic lupus erythematosus. *Am J Med*. 1992;93:513–519.
26. Roman MJ, Shanker BA, Davis A, Lockshin MD, Sammaritano L, Simantov R, Crow MK, Schwartz JE, Paget SA, Devereux RB, Salmon JE. Prevalence and correlates of accelerated atherosclerosis in systemic lupus erythematosus. *N Engl J Med*. 2003;349:2399–2406.
27. Ravenell RL, Kamen DL, Spence JD, Hollis BW, Fleury TJ, Janech MG, Almeida JS, Shaftman SR, Oates JC. Premature atherosclerosis is associated with hypovitaminosis D and angiotensin-converting enzyme inhibitor non-use in lupus patients. *Am J Med Sci*. 2012;344:268–273.
28. Sabio JM, Zamora-Pasadas M, Jiménez-Jáimez J, Albadalejo F, Vargas-Hitos J, Rodríguez del Aguila MD, Hidalgo-Tenorio C, Gonzalez-Gay MA, Jimenez-Alonso J, Alonso JJ. Metabolic syndrome in patients with systemic lupus erythematosus from Southern Spain. *Lupus*. 2008;17:849–859.
29. Sabio JM, Vargas-Hitos J, Zamora-Pasadas M, Mediavilla JD, Navarrete N, Ramirez A, Hidalgo-Tenorio C, Jáimez L, Martín J, Jiménez-Alonso J; Grupo Lupus Virgen de las Nieves. Metabolic syndrome is associated with increased arterial stiffness and biomarkers of subclinical atherosclerosis in patients with systemic lupus erythematosus. *J Rheumatol*. 2009;36:2204–2211.
30. Pons-Estel GJ, Alarcón GS, McGwin G Jr, Danila MI, Zhang J, Bastian HM, Reveille JD, Vilá LM; Lumina Study Group. Protective

effect of hydroxychloroquine on renal damage in patients with lupus nephritis: LXV, data from a multiethnic US cohort. *Arthritis Rheum*. 2009;61:830–839.
31. Tanay A, Leibovitz E, Frayman A, Zimlichman R, Shargorodsky M, Gavish D. Vascular elasticity of systemic lupus erythematosus patients is associated with steroids and hydroxychloroquine treatment. *Ann N Y Acad Sci*. 2007;1108:24–34.
32. Hurst NP, French JK, Gorjatschko L, Betts WH. Chloroquine and hydroxychloroquine inhibit multiple sites in metabolic pathways leading to neutrophil superoxide release. *J Rheumatol*. 1988;15:23–27.
33. Quintela AM, Jiménez R, Gómez-Guzmán M, Zarzuelo MJ, Galindo P, Sánchez M, Vargas F, Cogolludo A, Tamargo J, Pérez-Vizcaíno F, Duarte J. Activation of peroxisome proliferator-activated receptor-β/-δ (PPARβ/δ) prevents endothelial dysfunction in type 1 diabetic rats. *Free Radic Biol Med*. 2012;53:730–741.
34. Oelze M, Mollnau H, Hoffmann N, Warnholtz A, Bodenschatz M, Smolenski A, Walter U, Skatchkov M, Meinertz T, Münzel T. Vasodilator-stimulated phosphoprotein serine 239 phosphorylation as a sensitive monitor of defective nitric oxide/cGMP signaling and endothelial dysfunction. *Circ Res*. 2000;87:999–1005.
35. Sabio JM, Vargas-Hitos JA, Navarrete-Navarrete N, Mediavilla JD, Jiménez-Jáimez J, Díaz-Chamorro A, Jiménez-Alonso J; Grupo Lupus Virgen de las Nieves. Prevalence of and factors associated with hypertension in young and old women with systemic lupus erythematosus. *J Rheumatol*. 2011;38:1026–1032.
36. Budman DR, Steinberg AD. Hypertension and renal disease in systemic lupus erythematosus. *Arch Intern Med*. 1976;136:1003–1007.
37. Selzer F, Sutton-Tyrrell K, Fitzgerald S, Tracy R, Kuller L, Manzi S. Vascular stiffness in women with systemic lupus erythematosus. *Hypertension*. 2001;37:1075–1082.
38. Swaak AJ, van den Brink HG, Smeenk RJ, et al. Systemic lupus erythematosus: clinical features in patients with a disease duration of over 10 years, first evaluation. *Rheumatology (Oxford)*. 1999;38:953–958.
39. Petrin J, Rozman B, Dolenc P, Logar D, Bozic B, Vizjak A, Ferluga D, Jezersek P. The dissociation of arterial hypertension and lupus glomerulonephritis in systemic lupus erythematosus. *Blood Press*. 1993;2:108–112.
40. Meng XW, Feller JM, Ziegler JB, Pittman SM, Ireland CM. Induction of apoptosis in peripheral blood lymphocytes following treatment *in vitro* with hydroxychloroquine. *Arthritis Rheum*. 1997;40:927–935.
41. Huang X, Guo Y, Bao C, Shen N. Multidimensional single cell based STAT phosphorylation profiling identifies a novel biosignature for evaluation of systemic lupus erythematosus activity. *PLoS One*. 2011;6:e21671.
42. Okamoto A, Fujio K, Tsuno NH, Takahashi K, Yamamoto K. Kidney-infiltrating CD4+ T-cell clones promote nephritis in lupus-prone mice. *Kidney Int*. 2012;82:969–979.
43. Tang S, Lui SL, Lai KN. Pathogenesis of lupus nephritis: an update. *Nephrology (Carlton)*. 2005;10:174–179.
44. Alves JD, Ames PR. Atherosclerosis, oxidative stress and auto-antibodies in systemic lupus erythematosus and primary antiphospholipid syndrome. *Immunobiology*. 2003;207:23–28.
45. Bijl M. Endothelial activation, endothelial dysfunction and premature atherosclerosis in systemic autoimmune diseases. *Neth J Med*. 2003;61:273–277.
46. Alexánderson E, Ochoa JM, Calleja R, Juárez-Rojas JG, Prior JO, Jácome R, Romero E, Meave A, Posadas-Romero C. Endothelial dysfunction in systemic lupus erythematosus: evaluation with 13N-ammonia PET. *J Nucl Med*. 2010;51:1927–1931.
47. Lima DS, Sato EI, Lima VC, Miranda F Jr, Hatta FH. Brachial endothelial function is impaired in patients with systemic lupus erythematosus. *J Rheumatol*. 2002;29:292–297.
48. Johnson SR, Harvey PJ, Floras JS, Iwanochko M, Ibanez D, Gladman DD, Urowitz M. Impaired brachial artery endothelium dependent flow mediated dilation in systemic lupus erythematosus: preliminary observations. *Lupus*. 2004;13:590–593.
49. El-Magadmi M, Bodill H, Ahmad Y, Durrington PN, Mackness M, Walker M, Bernstein RM, Bruce IN. Systemic lupus erythematosus: an independent risk factor for endothelial dysfunction in women. *Circulation*. 2004;110:399–404.
50. Bengtsson C, Andersson SE, Edvinsson L, Edvinsson ML, Sturfelt G, Nived O. Effect of medication on microvascular vasodilatation in patients with systemic lupus erythematosus. *Basic Clin Pharmacol Toxicol*. 2010;107:919–924.
51. Ryan MJ, McLemore GR Jr. Hypertension and impaired vascular function in a female mouse model of systemic lupus erythematosus. *Am J Physiol Regul Integr Comp Physiol*. 2007;292:R736–R742.
52. Kelley VR, Wüthrich RP. Cytokines in the pathogenesis of systemic lupus erythematosus. *Semin Nephrol*. 1999;19:57–66.
53. Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circ Res*. 2000;87:840–844.

Novelty and Significance

What Is New?

- We found for the first time that reactive oxygen species levels are increased in aorta from systemic lupus erythematosus mice and chronic hydroxychloroquine treatment improves endothelium-dependent relaxation, essentially by preserving the nitric oxide–mediated component.
- This protective effect may be attributable to a decrease in the vascular oxidative stress by normalizing the expression of nicotinamide adenine dinucleotide phosphate oxidase subunits.

What Is Relevant?

- These results reinforce the notion that hydroxychloroquine should be done not only to patients with mild systemic lupus erythematosus disease, but also to patients with major organ involvement.

Summary

Chronic hydroxychloroquine treatment reduces systolic blood pressure and improves endothelial dysfunction, essentially by preserving the nitric oxide–mediated component, in severe lupus mice. This protective effect may be attributable to a decrease in the vascular oxidative stress.