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\textsuperscript{\textsubscript{G}}-nitro-\textsuperscript{\textsubscript{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;64:00-00.) • 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, which are major causes of mortality in these patients. 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. Indeed, SLE is characterized by a high incidence of hypertension, 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 ($O_2^-$) play a critical role in the pathogenesis of endothelial dysfunction, an early event in most cardiovascular diseases, including hypertension. Reactive oxygen species (ROS) have been considered as risk and enhancer factors for autoimmune diseases, 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. Free radical–mediated reactions are implicated in endothelial dysfunction in SLE, and renal oxidative stress plays an important mechanistic role in the development of autoimmune-mediated hypertension.
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. Hydroxychloroquine has been shown to reduce serum cholesterol and low-density lipoprotein levels compared with patients treated with corticosteroids, and the lipid-lowering effect of hydroxychloroquine has significantly lower mean glucose levels and markers of insulin resistance in women with SLE. Hydroxychloroquine was protective against thrombo-vascular events, and a negative relationship was found between the use of hydroxychloroquine and the presence of atherosclerosis. Hydroxychloroquine has also been inversely associated with the presence of metabolic syndrome and subclinical arteriosclerosis in SLE. Again, protective effect of hydroxychloroquine in retarding renal damage in SLE is also evident. 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, suggesting the involvement of other protective mechanisms. Hydroxychloroquine inhibited O·⁻ generation in mononuclear phagocytes stimulated by different agents, 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 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-phenyl-indole dichlorohydrate (DAPI) fluorescence in sections of unfixed thoracic aortic rings incubated with dihydroethidium and counter-stained 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...
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 (crecent 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 (crecent), 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.
were incubated in the presence of SLE-Veh and Ctrl-Veh groups were found when the rings responses in this contractile response to phenylephrine between nitro-

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6.8±0.6 mN; control treated with hydroxychloroquine (maximal effect, 6.5±0.4 mN; as compared with SLE mice (maximal effect, 9.7±0.8 mN) as compared from SLE mice (maximal effect, 56.0±5.6%; vehicle-treated SLE mice (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$_2^-$ scavenger) and pegylated superoxide dismutase. The inhibitory effect of both O$_2^-$ scavengers suggests that the primary source of oxidant stress is likely to be O$_2^-$.

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$^{	ext{phox}}$ (Figure 5C), without change of p22$^{	ext{phox}}$ (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. 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$^\text{-}$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$^{-4}$ 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.
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...
cular reactivity to local heat or iontophoretically administered SLE.46 In large vessels, flow-mediated dilatation is impaired of cardiovascular disease and is also a principal element of endothelial damage or dysfunction. Endothelial cell dysfunction represents the earliest indication of the development of proteinuria and increased blood pressure, suggesting that the impaired response to acetylcholine begins before the development of proteinuria 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 intimal 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.47,48 However, the cutaneous microvascular reactivity to local heat or iontophoretically administered acetylcholine did not differ between patients with SLE and matched controls.49 However, in NZBWF1 mice, which have uniform genetics and environmental conditions, we found impaired aortic endothelium-dependent relaxation response to acetylcholine. Interestingly, Ryan and McLemore45 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.40 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.53 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-eGMP 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$_2^-$, which reacts rapidly with and inactivates NO.54 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 gp91phox or its vascular homologous NOX-1 and NOX-4, rac, p22phox, p47phox, and p67phox, is considered the major source of O$_2^-$ in the vascular wall. We found a marked increase in aortic NADPH oxidase activity in SLE mice, accompanied with an increase in mRNA of p47phox and NOX-1, being without effect on p22phox. Hydroxychloroquine and antioxidant treatments inhibited the upregulation of these subunits in SLE mice. A previous in vitro study reported that hydroxychloroquine inhibited O$_2^-$ production on human polymorphonuclear neutrophils stimulated by opsonized zymosan, phorbol myristate acetate, or fluorode.52 Herein, we show that hydroxychloroquine not only inhibits the activity, but also the expression of NADPH oxidase, the main vascular source of O$_2^-$.

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, our data suggest increased NO bioavailability after both treatments. Taken together, the results suggest that the reduction of $\text{O}_2^-$ 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 anti-aggregant 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.

**Sources of Funding**

This work was supported by grants from Comisión Interministerial de Ciencia y Tecnología (SAF2010-22606-C02-01, SAF2011-28150), Junta de Andalucía (Proyecto de excelencia, PI2-CTS-2722), Ministerio de Ciencia e Innovación (Circuitos de Excelencia, CIR12/0042/0011 and CIR12/0042/0040), Spain. M.J. Zarzuelo is a holder of studentship from Spanish Ministry of Science and Education.

**Disclosures**

None.

**References**


**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 mouse. This protective effect may be attributable to a decrease in the vascular oxidative stress.
Chronic Hydroxychloroquine Improves Endothelial Dysfunction and Protects Kidney in a Mouse Model of Systemic Lupus Erythematosus

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Hypertension. published online May 19, 2014;
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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http://hyper.ahajournals.org/content/early/2014/05/19/HYPERTENSIONAHA.114.03587

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CHRONIC HYDROXYCHLOROQUINE IMPROVES ENDOTHELIAL DYSFUNCTION AND PROTECTS KIDNEY IN A MOUSE MODEL OF SYSTEMIC LUPUS ERYTHEMATOSUS.

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Short Title: Hydroxychloroquine in SLE hypertension.

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Materials and Methods

Animals and experimental groups

The investigation conforms to the National Institutes of Health (NIH) 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 HCQ (10 mg/kg/day) or vehicle (1mL of 1% methylcellulose), or a combination of the antioxidants tempol (2.0 mmol/L) and apocynin (1.5 mmol/L) in the drinking water (1) for 5 weeks. We used a high dose of HCQ, as compared to that used in human (200-400 mg/day), but for a short time period. Urine was collected and assessed for the presence of proteinuria, as described previously (2). Only mice with no sign of albuminuria at 30 weeks of age were included in the study. Animals were maintained at a constant temperature (24 ± 1 ºC), with a 12-hour light/dark cycle, on a standard chow and water ad libitum. Mice were randomly divided into five groups: control-untreated (Ctrl-Veh), control treated with HCQ (Ctrl-HCQ), SLE-untreated (SLE-Veh), SLE treated with HCQ (SLE-HCQ), and SLE treated with tempol and apocynin (SLE-Antiox).

Anti-dsDNA and malonyldialdehyde

Plasma anti-dsDNA antibodies were measured as described previously (3). Only NZBWF1 mice with positive anti-ds-DNA antibodies (at values ≥ 1 SD from control) were considered to have SLE. Plasma malonyldialdehyde (MDA) content was evaluated as previously described (4). Briefly, 100 µl of plasma reacted with a chromogenic reagent, 1-methyl-2-phenylindole (10.3 mmol/L) in acetonitrile and 37% aqueous HCl (10.4 M). After incubation of the reaction mixture for 40 minutes in a 45ºC water bath, the absorbance was measured at 586 nm in a GBC 920 spectrophotometer.

Blood pressure measurements

Systolic blood pressure (SBP) was measured in conscious mice by tail-cuff plethysmography (Digital Pressure Meter LE 5001, Panlab) as described previously (2). Briefly, mice were held in a plastic tube, and their tail was put through a rubber cuff, and the cuff was inflated with air. The pressure level at which the first pulse appeared, after blood flow had been interrupted with the inflated cuff, was designated SBP. At least seven determinations were made in every session and the mean of the lowest three values within 5 mmHg was taken as the SBP. At the end of the experimental period, a polyethylene catheter containing 100U heparin in isotonic, sterile, NaCl solution was inserted into the left carotid artery in some mice. Twenty-four hours after implantation of the catheter, intraarterial BP and heart rate (HR) were recorded continuously for 60 min with a sampling frequency of 400/s (McLab; AD Instruments, Hastings, UK). BP and HR values obtained during the last 30 min were averaged for intergroup comparisons.

Physical characteristics, cardiac and renal weight indices

Body weight (in grams) was measured for all the mice. At the end of the experimental period, the animals were euthanized and dissected. The hearts were excised; the atria and the right ventricle were then removed and the remaining left ventricles weighed.
The left ventricular and kidney weight indices were calculated by dividing the left ventricle and kidney weight by the tibia length.

Assessment of renal injury. Proteinuria

Mice were placed in metabolic cages to collect urine for 24 h. When the experimental period was completed, all mice were housed in metabolic cages with free access to food and their respective drinking fluids, and treatments were continued for 4 days (2 days for adaptation and 2 experimental days) to collect urine samples. Proteinuria was determined as previously described (5), using bovine serum albumin as standard and the results were expressed as mg of protein excreted, normalized by body weight (per 100 g of mice), and time (during 24 h). The means of the values obtained during the 2 experimental days were used for statistical analyses between groups.

Histopathological techniques

Kidney biopsies were fixed in 10% buffered formalin for 24 hrs, and embedded in paraffin. Then, 4-µm sections were cut along the central axis of the biopsies and dewaxed and hydrated for staining with hematoxylin-eosin, Peryodic Acid Schiff, and Masson’s trichrome. The presence of SLE-like lesions, such as glomerular sclerosis, mesangial proliferation, glomerular hypercelularity, extracapillar proliferation (crescent formation), capillary wire-loop lesion, fibrinoid necrosis in glomerular capillary and arterioles, interstitial inflammatory infiltrate and tubular casts were studied. Morphological study on light microscopy was done in a blinded fashion (MGM and FO). Results were calculated semiquantitatively using a 0 to 3 scale (0, absence; 1, mild [<10% of glomeruli or tubules injured]; 2, moderate [10 to 25%]; 3, severe [>25%]) (2).

Vascular contractility in vitro

Descending thoracic aortic rings were dissected from animals and were suspended in a wire myograph (model 610M, Danish Myo Technology, Aarhus, Denmark) for isometric tension measurement as previously described (6). The organ chamber was filled with Krebs solution (composition in mM: NaCl 118, KCl 4.75, NaHCO₃ 25, MgSO₄ 1.2, CaCl₂ 2, KH₂PO₄ 1.2 and glucose 11) at 37 °C and gassed with 95% O₂ and 5% CO₂ (pH ∼ 7.4). Length–tension characteristics were obtained via the myograph software (Myodaq 2.01) and on the basis of these, aorta arteries were loaded to a tension equivalent to 0.5 g.

In endothelium-intact aorta, cumulative concentration-response curves to phenylephrine (10⁻⁹ M-10⁻⁶ M) were performed in the absence or in the presence of N⁶-nitro-L-arginine methyl ester (L-NAME, 100 µM), or tempol (1 µM). The concentration-relaxation response curves to acetylcholine (10⁻⁸ M-10⁻⁶ M) were performed in intact rings precontracted by phenylephrine to obtain similar level of precontraction in both control or L-NAME treated aortic rings. The relaxant responses to sodium nitroprusside (10⁻⁹ M-10⁻⁵ M) were studied in the dark in endothelium-denuded vessels precontracted by phenylephrine (10⁻⁵ M). Relaxant responses to acetylcholine and sodium nitroprusside were expressed as a percentage of precontraction induced by phenylephrine. Aorta was denuded of endothelium by gently rubbing the intimal surface with a needle. The
absence of functional endothelium was tested by observing no relaxant response to acetylcholine (10^{-6} M).

**In situ detection of vascular ROS levels**

Unfixed thoracic aortic rings were cryopreserved (phosphate buffer solution 0.1 mol/L, PBS, plus 30% sucrose for 1-2h), included in optimum cutting temperature compound medium (Tissue-Tek; Sakura Finetechincal, Tokyo, Japan), frozen (-80ºC), and 10 μm cross sections were obtained in a cryostat (Micron International Model HM500 OM). Sections were incubated for 30 min in Hepes buffered solution containing dihydroethidium (DHE, 10^{-5} M), counterstained with the nuclear stain 4,6-diamidino-2-phenylindole dichlorohydrate (DAPI, 3 x 10^{-7} M) and in the following 24 h examined on a fluorescence microscope (Leica DM IRB, Wetzlar, Germany). Representative DHE staining fields of each slide were photographed at x 400 magnification, and ethidium and DAPI fluorescence were quantified using ImageJ (version 1.32j, NIH, http://rsb.info.nih/ij/). ROS level was estimated from the ratio of ethidium/DAPI fluorescence (2).

**Vascular NADPH oxidase activity**

The lucigenin-enhanced chemiluminescence assay was used to determine NADPH oxidase activity in intact aortic rings, as previously described (4). Aortic rings from all experimental groups were incubated for 30 minutes at 37 ºC in HEPES-containing physiological salt solution (pH 7.4) of the following composition (in mM): NaCl 119, HEPES 20, KCl 4.6, MgSO4 1, Na2HPO4 0.15, KH2PO4 0.4, NaHCO3 1, CaCl2 1.2 and glucose 5.5. Aortic production of O2^{-} was stimulated by addition of NADPH (100 μM). Rings were then placed in tubes containing physiological salt solution, with or without NADPH, and lucigenin was injected automatically at a final concentration of 5 μM to avoid known artifacts when used a higher concentrations. NADPH oxidase activity was determined by measuring luminescence over 200 s in a scintillation counter (Lumat LB 9507, Berthold, Germany) in 5-s intervals and was calculated by subtracting the basal values from those in the presence of NADPH. Vessels were then dried, and dry weight was determined. NADPH oxidase activity is expressed as relative luminescence units (RLU)/min/mg dry aortic tissue.

**Flow Cytometry**

Spleens were collected from mice. The tissues were smashed with wet slides very well to decrease friction and then the solutions were filtered through a cell strainer of 70μM. Cells were isolated followed by lysis of red blood cells with Gey’s solution. 1×10^6 cells were counted and submitted to FcγR blocking by treatment with the incubation in a plate prepared one day in advance with a solution of anti-CD3 (clone17A2, eBioscience) and anti-CD28 (clone 37.51, eBioscience). After 24 hours, a protein transport inhibitor (BD GolgiPlug™), was added to the plate for an optimum detection of intracellular cytokines by flow cytometry. After that, the cells were transferred to polystyrene tubes for the surface staining with mAbs anti-CD4 (PerCP-Cy™, clone...
RM4-5 BD Pharmigen™ and anti-B220 (APC, clone RA3-6B2, BD Pharmigen™) for 15 min at 4°C in the dark. The splenocytes were then fixed, permeabilized with the Fix/Perm Fixation/Permeabilization kit (eBioscience) and intracellular staining was made with mAbs anti-Foxp3 (PE,clone FJK-16s, eBioscience), anti-IL-17A (PE-Cy7, clone eBio17B7, eBioscience) and anti-IFNγ (Alexa Fluor® 488, clone XMG1.2, eBioscience) for 30 min at 4°C in the dark. Data collection was performed using a flow cytometer CANTO II (BD Biosciences).

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

For RT-PCR analysis, total RNA was extracted from the aorta by homogenization and converted to cDNA by standard methods. Polymerase chain reaction was performed with a Techne Techgene thermocycler (Techne, Cambridge, UK). A quantitative real-time RT-PCR technique was used to analyze mRNA expression of eNOS, p22phox, p47phox, and NOX1. The sequences of the sense and antisense primers used for amplification are described in Table S1. Preliminary experiments were carried out with various amounts of cDNA to determine nonsaturating conditions of PCR amplification for all the genes studied. Therefore, under these conditions, relative quantification of mRNA was assessed by the RT-PCR method used in this study. The efficiency of the PCR reaction was determined using a dilution series of a standard vascular sample. Quantification was performed using the ∆∆Ct method. The housekeeping gene β-actin was used for internal normalization (4).

Western blotting analysis

Aortic homogenates were run on a sodium dodecyl sulphate (SDS)-polyacrilamide electrophoresis. Proteins were transferred to polyvinylidene difluoride membranes (PVDF), incubated with primary monoclonal mouse anti-phospho- vasodilator-stimulated phosphoprotein (VASP)-Ser239 antibody (Calbiochem, Darmstadt, Germany) overnight and with the correspondent secondary peroxidase conjugated antibody. Antibody binding was detected by an ECL system (Amersham Pharmacia Biotech, Amersham, UK) and densitometric analysis was performed using Scion Image-Release Beta 4.02 software (http://www.scioncorp.com) (4). Samples were re-probed for expression of smooth muscle α-actin.

Statistical analysis

Results are expressed as means ± SEM. Statistical analyses were performed using Graph Pad Prism 5 software. A two-factor ANOVA was used to test for drug or group interactions. When a significant interaction was detected, one-way ANOVA with a Student-Neuman-Keuls post hoc test was used to discern individual differences between groups. Significance was accepted at p<0.05.
References


### Table S1. Oligonucleotides for real-time RT-PCR

<table>
<thead>
<tr>
<th>mRNA targets</th>
<th>Descriptions</th>
<th>Sense</th>
<th>Antisense</th>
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<tr>
<td>p47phox</td>
<td>p47phox subunit of NADPH oxidase</td>
<td>ATGACAGCCAGTGAGAAGAG</td>
<td>CCGATAGGTCTGAAGGCTGTAG</td>
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<td>p22phox</td>
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<td>eNOS</td>
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<td>Actb</td>
<td>Beta actin</td>
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<td>ATGCCACAGGATTCCATACC</td>
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Table S2. Effects on renal injury.

<table>
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<tr>
<th>Variables</th>
<th>Ctrl-Veh group (n = 10)</th>
<th>Ctrl-HCQ group (n = 16)</th>
<th>SLE-Veh group (n = 11)</th>
<th>SLE-HCQ group (n = 12)</th>
<th>SLE-Antiox group (n = 6)</th>
<th>p-values</th>
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<tbody>
<tr>
<td>Mesangial hyalinosis</td>
<td>0.50±0.23</td>
<td>0.60±0.23</td>
<td>1.72±0.24</td>
<td>0.75±0.18</td>
<td>1.33±0.36</td>
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<td>Crescents</td>
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<td>0.81±1.50</td>
<td>0.08±0.08</td>
<td>0.33±0.23</td>
<td>0.017*†‡</td>
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<td>Hyalin trombi</td>
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<td>0.20±0.21</td>
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<td>0.25±0.26</td>
<td>0.16±0.18</td>
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<tr>
<td>Wire-loop</td>
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<td>0.54±0.25</td>
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<td>Glomerular hypercellularity</td>
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<td>0.90±0.29</td>
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<td>Glomerular fibrinoid necrosis</td>
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<td>Tubular casts</td>
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<td>Vascular fibrinoid necrosis</td>
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<td>0.00±0.00</td>
<td>0.036±0.16</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.002*†‡</td>
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</tbody>
</table>

Values are expressed as mean±standard error. KW: Kruskal-Wallis test; *p<0.05 Mann-Whitney U-test systemic lupus erythematosus (SLE) group vs SLE-hydroxychloroquine (HCQ) group; †p<0.05 Mann-Whitney U-test SLE group vs control (Ctrl)-HCQ group; ‡p<0.05 Mann-Whitney U-test SLE group vs Ctrl group; §p<0.05 Mann-Whitney U-test antioxidant (SLE-Antiox) group vs SLE-HCQ group.
**Figure S1.** Effects of chronic Hydroxychloroquine (HCQ) and antioxidant (Antiox; tempol + apocynin) treatment on total T (CD4+) (A) and B (B220+) (B) lymphocytes, and Treg (CD4+ FOXP3+), Th17 (CD4+ IL17+), and Th1 (CD4+ IFNγ+) cells (C) measured in spleens from all experimental groups measured by flow cytometry. Experimental groups: Control (n = 5), SLE (Systemic lupus erythematosus, n = 5), SLE-HCQ (10 mg/kg/day, n = 5), SLE-Antiox (n = 5). Values are expressed as mean ± SEM (n = 5). **p<0.01 and *p<0.05 vs control group. #p<0.05 vs SLE group.
Figure S2. Effects of chronic Hydroxychloroquine (HCQ) and antioxidant (Antiox; tempol + apocynin) treatment on plasma malonyldialdehyde (MDA) from control and systemic lupus erythematosus (SLE) mice. Experimental groups: Control (n = 8), SLE (Systemic lupus erythematosus, n = 7), SLE-HCQ (10 mg/kg/day n = 8), SLE-Antiox (n = 7). Values are expressed as mean ± SEM (n = 7-8). **p<0.01 vs control group. ##p<0.01 and #p<0.05 vs SLE group.