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Autoimmune therapeutic chloroquine lowers blood pressure and improves endothelial function in spontaneously hypertensive rats

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Abstract

It has been suggested that hypertension results from a loss of immunological tolerance and the resulting autoimmunity may be an important underlying factor of its pathogenesis. This stems from the observations that many of the features involved in autoimmunity are also implicated in hypertension. Furthermore, the underlying presence of hypertension and cardiovascular disease are frequently observed in patients with autoimmune diseases. Antimalarial agents such as chloroquine are generally among the first line treatment options for patients with autoimmune diseases; however, whether they can improve a hypertensive phenotype in a genetic model of essential hypertension remains to be clarified. Therefore, we hypothesized that chloroquine treatment would improve endothelial function and lower blood pressure in spontaneously hypertensive rats (SHR). We treated adult SHR and Wistar-Kyoto rats (12 weeks old), as well as a group of young SHR (5 weeks old), with chloroquine (40 mg/kg/day via intraperitoneal injection) for 21 days. Chloroquine lowered blood pressure in adult SHR, but did not impede the development of high blood pressure in young SHR. In isolated mesenteric resistance arteries from SHR of both ages, chloroquine treatment inhibited cyclooxygenase-dependent contraction to acetylcholine, lowered vascular and systemic generation of reactive oxygen species, and improved nitric oxide bioavailability. Overall, these data reveal the anti-hypertensive mechanisms of chloroquine in the vasculature, which may be important for lowering risk of cardiovascular disease in patients with autoimmune diseases. Furthermore, it adds to the growing body of evidence suggesting that autoimmunity underlies hypertension.

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CONFLICT OF INTEREST

None.

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Graphical abstract

Keywords

Chloroquine; autoimmunity; endothelial function; hypertension

1. INTRODUCTION

Essential hypertension and endothelial dysfunction are manifested by systemic and local (vascular) inflammation [1] and immune system participation in the pathogenesis of hypertension has been known for a number of years [2]. Therefore, some authors have suggested that in some cases, hypertension is a result of an autoimmune reaction [3, 4]. The possibility that a loss of immunological tolerance possibility underlies hypertension has increased in recent years with the reports that self-antigens (e.g., neo-antigens or damage-associated molecular patterns) [5] and B cells [6] are present in hypertensive patients and animals. In addition, several autoimmune disorders, such as rheumatoid arthritis and systemic lupus erythematosus (SLE) are characterized by cardiovascular complications such as hypertension and endothelial dysfunction [7, 8]. Antimalarial agents such as chloroquine or hydroxychloroquine have long been used in the treatment of autoimmune diseases [9, 10], and at present, they remain among one of the first-line therapeutic options [11]. The efficacy of these agents in autoimmune conditions has been attributed in various mechanisms including photoprotection, lysosomal stabilization, suppression of antigen presentation to T cells, and inhibition of prostaglandin and cytokine synthesis [12].

Clinically, patients with lupus treated with hydroxychloroquine present a lower prevalence of thromboembolic events [13, 14] and chronic treatment with hydroxychloroquine reduced hypertension and improved endothelium-dependent relaxation in conduit vessels from SLE mice [15]. These data suggest that antimalarial treatments such as chloroquine and hydroxychloroquine may have beneficial effects on cardiovascular function associated with autoimmune diseases. However, the effects of chloroquine on cardiovascular function in essential hypertension have not been previously addressed. Therefore, given that

hypertension underlies many autoimmune conditions [7] and that dysfunction of the immune system is involved in human and experimental hypertension (e.g., pro-inflammatory cytokines, T cell activation, autoantibodies) [3, 4], we hypothesized that chloroquine treatment would lower blood pressure and improve endothelial function in spontaneously hypertensive rats (SHR), a genetic model of essential hypertension.

2. MATERIALS AND METHODS

2.1 Animals

Male SHR and normotensive Wistar-Kyoto (WKY) rats were used for this investigation (Envigo, Indianapolis, IN, USA). Adult SHR and WKY were 12 weeks of age at the onset of treatment. A younger group of SHR was also used and these rats were 5 weeks of age at the onset of treatment. We treated rats of two different ages (young and adult) to examine if chloroquine treatment had differential effects during the developmental and maintenance phases of hypertension. The sample size indicated per experiment (see figure legends) is the number of independent rats used, respective of strain and treatment group.

All rats were maintained on a 12:12 h light-dark cycle with both chow and water *ad libitum*. All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals and were reviewed and approved by the Institutional Animal Care and Use Committee of Augusta University. Rats were killed by thoracotomy and exsanguination via cardiac puncture under isoflurane anesthesia (5% in 100% O₂ administered via nose cone). Tissues were subsequently harvested.

2.2 Treatment

Rats were randomly assigned to receive either 40 mg/kg/day of lysosomotropic agent chloroquine (Sigma-Aldrich, St Louis, MO, USA) or vehicle (Veh; saline), administered via intraperitoneal injection for 21 days starting as 12 (adult) or 5 (young) weeks of age. This dose of chloroquine was chosen based on the investigation by Long *et al.* [16] who observed beneficial effects on indices of pulmonary hypertension at 25 and 50 mg/kg/day.

2.3 Blood pressure and heart rate

Systolic blood pressure (SBP) and heart rate were measured in conscious rats via tail cuff before treatment (day 0) and 24 h after the last treatment injection (day 22) using a RTBP1001 blood pressure system (Kent Scientific Corporation, Torrington, CT, USA). An average of the SBP and heart rate from 10 cycles was taken from each animal and then averaged within group. The choice of tail cuff methodology for blood pressure measurements was reasoned due to its non-invasive properties, and thus limiting immune system activation due to surgery and unintended tissue injury.

2.4 Sodium excretion, urine volume, and food and water consumption

Excreted urine and food and water consumption were measured for a 24 h period immediately following the final treatment injection. Rats were housed in individual metabolic cages (Ancare, Bellmore, NY, USA) that prevented food and fecal contamination of urine samples. One week prior to the collection period, rats were allowed a 24 h period to

acclimatize to the metabolic cages. Food and water intakes during the collection period were available *ad libitum*. Urinary sodium concentration was determined by atomic absorption spectrophotometry (PerkinElmer, Waltham, MA, USA).

2.5 Vascular function

Third order mesenteric resistance arteries (MRA) and aortic segments were mounted on DMT wire and pin myographs (Danish MyoTech, Aarhus, Denmark), respectively. Mesenteric resistance arteries were normalized to their optimal lumen diameter for active tension development, as described previously [17, 18]. Aortic segments were set to a passive force of 30 mN. All arteries were initially contracted with 120 mmol/L potassium chloride (KCl). Endothelium integrity was then tested with phenylephrine-induced contraction (3×10^{-6} mol/L) followed by endothelium-dependent relaxation with acetylcholine (ACh; 3×10^{-6} mol/L).

Mesenteric resistance artery function was assessed by performing cumulative concentration-response curves to ACh (10^{-9} – 10^{-5} mol/L) and nitric oxide (NO) donor sodium-nitroprusside (SNP; 10^{-9} – 3×10^{-5} mol/L), after an initial contraction with 3×10^{-6} mol/L norepinephrine (NE) (all Sigma-Aldrich). Aortic vascular function was assessed via NE cumulative concentration-response curves (10^{-11} – 10^{-5} mol/L). Thirty minutes prior to concentration-response curves, some arteries were incubated with inhibitors of various vasoactive molecules. In MRA these inhibitors included, indomethacin [cyclooxygenase (COX) inhibitor, 10^{-5} mol/L] (Sigma-Aldrich), N^G -nitro-L-arginine [NO synthase (NOS) inhibitor, L-NNA; 10^{-4} mol/L] (Sigma-Aldrich), or tempol [superoxide dismutase (SOD) mimetic, 10^{-3} mol/L] (Tocris, Ellisville, MO, USA). In aorta, L-NNA (10^{-4} mol/L) was used. Relaxation responses to ACh and SNP are presented as a percent of the NE pre-contraction and contractile responses to NE are presented as a percent of the maximum response to KCl.

2.6 Immunoblotting

Mesenteric resistance arteries were cleaned of perivascular adipose tissue, snap frozen in liquid nitrogen, and then homogenized in ice-cold tissue protein extraction reagent (T-PER) (Thermo Fisher Scientific, Waltham, MA, USA), with protease inhibitors (sodium orthovanadate, phenylmethanesulfonylfluoride, and protease inhibitor cocktail) and phosphatase inhibitors (sodium fluoride and sodium pyrophosphate) (all Sigma-Aldrich). Equal amounts of protein (30–50 μ g) were loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (8–12%) for Western blotting. Polyvinylidene difluoride or nitrocellulose membranes were probed for the expression of COX 1, COX 2, phosphorylated endothelial NOS (eNOS)^{Ser1177}, total eNOS, and Cu/Zn SOD (further details on Western blotting are provided in supplementary table 1). Phosphorylated protein expression was normalized to total protein expression; all other proteins were normalized to β actin. Densitometric analysis was performed by Un-Scan-It software (Version 6.1) (Silk Scientific, Orem, UT, USA).

2.7 Thromboxane measurement

Due to the short lived nature of thromboxane A₂, thromboxane B₂ was measured as an estimate of *in vivo* thromboxane A₂ production [19]. Briefly, MRA were cleaned of perivascular adipose tissue and punctured to allow the release of prostanoids from the artery lumen. After incubating MRA in 200 µl physiological salt solution (PSS) for 5 min, the supernatant was collected and snap frozen in liquid nitrogen. Thromboxane B₂ was then measured in the supernatant using a competitive ELISA kit and according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI, USA).

2.8 Prostacyclin measurement

Due to the short lived nature of prostacyclin, 6-keto prostacyclin F_{1α} was measured as an estimate of *in vivo* prostacyclin production [20]. Briefly, arterial blood was collected from the abdominal aorta in tubes containing EDTA. Blood was immediately centrifuged at 1500 g for 15 minutes at 4°C. Plasma was stored in multiple aliquots at -80°C until analysis. 6-keto Prostacyclin F_{1α} was then measured in plasma using a competitive ELISA kit and according to the manufacturer's instructions (Cayman Chemical).

2.9 Arterial nitric oxide measurement

Due to the short lived nature of NO, nitrite (NO₂⁻) and nitrate (NO₃⁻) were measured as an estimate of *in vivo* NO production [21]. Briefly, cleaned and punctured MRA were incubated in 200 µl PSS and stimulated with ACh (10⁻⁶ mol/L) for 5 min. The supernatant was collected and snap frozen in liquid nitrogen. NO₂⁻/NO₃⁻ was then measured in the supernatant using colorimetric assay and according to the manufacturer's instructions (Cayman Chemical).

2.10 Systemic oxidative stress measurement

Malondialdehyde (MDA) was measured in plasma using a Thiobarbituric Acid Reactive Substances (TBARS) Assay Kit according to the manufacturer's instructions (Cayman Chemical).

2.11 Gelatin zymography

To assess arterial remodeling enzyme activity, gelatin zymography was performed for matrix metalloproteinase (MMP)2, as described previously [22, 23]. Briefly, aortas were cleaned and homogenized as described above for Western blots. Equal amounts of protein (20 µg) were loaded into SDS-PAGE gels (0.1% gelatin) and separated by electrophoresis. Gels were then washed with 2.5% Triton X-100 and then incubated in zymogram development buffer at 37°C for 23 h. Following development, gels were stained overnight at room temperature with Coomassie Brilliant Blue stain (Bio-Rad, Hercules, CA, USA) and then destained for 4 h at room temperature using Coomassie Destain Solution (Bio-Rad). Recombinant MMP2 standard (EMD Millipore, Billerica, MA) was used as a positive control. Gels were quantified by densitometric analysis using Un-Scan-It software (Version 6.1) (Silk Scientific).

2.12 Statistical analysis

The statistical procedures used included Student's t-test, one-way and two-way analysis of variance (ANOVA), and non-linear regression analysis (LogEC₅₀ and E_{max}). Tukey's *post-hoc* testing was used in all cases using a one-way ANOVA and the Bonferroni *post-hoc* was used in all cases using a two-way ANOVA. All analyses were performed using the data analysis software GraphPad Prism 5.0 (La Jolla, CA, USA). Data are presented as mean $\pm$ S.E.M and statistical significance was set at $p < 0.05$.

3. RESULTS

Chloroquine treatment significantly lowered SBP in adult SHR, but did not impede the development of high SBP in young SHR or lower SBP in normotensive WKY (Figure 1A). Chloroquine treatment did not change heart rate in any group (Figure 1B). In adult SHR and WKY, chloroquine decreased body mass after the third week of treatment (Supplementary figure 1A). After normalizing the loss of body mass with tibia length, chloroquine did not change total heart mass, left ventricle (LV) mass, or right ventricle (RV) mass (Supplementary figure 1B–G). However wet to dry lung mass, indicative of lung edema, was lower in adult SHR after chloroquine treatment (Supplementary figure 1H). In young SHR, chloroquine did not influence body mass gain (Supplementary figure 1I).

To determine if the decrease in blood pressure in adult SHR was associated with the decreased body weight due an anorexic effect of chloroquine, urinary sodium excretion, as well as food and water consumption, were measured with metabolic cages. We observed that urinary sodium excretion was increased significantly in adult SHR treated with chloroquine and this was coupled with increased food consumption (Table 1). Therefore, the decrease in blood pressure caused by chloroquine treatment in SHR cannot be attributed to lower sodium intake, and, if anything, it is possible that their greater sodium intake could have attenuated the blood pressure-lowering effect that was measured.

As the anti-hypertensive effects of chloroquine did not appear to be mediated via changes in sodium balance, endothelium-dependent and -independent relaxation was assessed in isolated MRA. Chloroquine abolished the contractile responses to high concentrations of ACh in adult and young SHR (Figure 2C and 2E). No group or treatment differences were observed in endothelium-independent relaxation (SNP concentration-responses curves) for both ages of SHR (Figure 2D and 2F). Chloroquine did not affect the ACh- or SNP-induced relaxation in WKY (Figure 2A and 2B).

In hypertensive arteries, it has been well established that ACh is able to induce contractile responses as a result of COX-derived contractile factors [24]. To test that chloroquine suppresses contraction to ACh via inhibition of the COX enzymes, some arteries were incubated with the COX inhibitor indomethacin prior to concentration-response curves with ACh. In Veh- and chloroquine-treated WKY, indomethacin had no effect on the MRA relaxation to ACh (Figure 3A and 3B). In MRA from Veh-treated SHR, the contractile response to ACh was abolished after incubation with indomethacin in both adult and young (Figure 3E and 3K). However, in MRA from chloroquine-treated SHR, there was no difference in the contractile response to ACh, with or without indomethacin, in both adult

and young (Figure 3F and 3L). Chloroquine also significantly lowered protein expression of both COX 1 (Figure 3M) and COX 2 (Figure 3N) in MRA from young SHR, but not adult SHR (Figure 3G and 3H) or WKY (Figure 3C and 3D). This indicates that in MRA from young SHR, chloroquine diminished expression and activity of the COX enzymes, whereas in MRA from adult SHR, chloroquine only decreased the activity of the enzymes. To corroborate the conclusion that MRA from chloroquine-treated SHR had decreased activity of the COX enzymes, despite no change in expression, MRA were incubated in PSS and the supernatant was collected for thromboxane B₂ measurements. Thromboxane B₂ production was significantly reduced in MRA from chloroquine-treated adult SHR (Figure 3I). On the other hand, while plasma levels of 6-keto prostaglandin F_{1α} were decreased in SHR compared to WKY, its expression was not changed by chloroquine treatment in either strain (Figure 3J). These results indicate that chloroquine caused a reduction in the activity of the COX enzymes in adult SHR, supporting our concentration-response curves to ACh with and without indomethacin.

To evaluate the ability of chloroquine to restore arterial NO in SHR, some MRA were incubated with the NOS inhibitor L-NNA prior to concentration-response curves with ACh. In MRA from WKY, incubation with L-NNA decreased the sensitivity to ACh in both Veh- and chloroquine-treated groups (Figure 4A and 4B). In MRA from Veh-treated SHR, there was no difference in the response to high concentrations of ACh ($>10^{-7}$ mol/L), with or without L-NNA, in both adult and young (Figure 4C and 4M). However in MRA from chloroquine-treated SHR, there was a significant difference in the response to high concentrations of ACh ($>10^{-7}$ mol/L), with or without L-NNA, in both adult and young (Figure 4D and 4N). To corroborate the conclusion that MRA from chloroquine-treated adult SHR had more arterial NO, MRA were incubated in PSS and stimulated with ACh (10^{-6} mol/L) for 5 min. The supernatant was then collected for combined NO₂⁻ and NO₃⁻ measurement. We observed that NO₂⁻/NO₃⁻ levels were increased in MRA from chloroquine-treated adult SHR (Figure 4E), although chloroquine did not change unstimulated expression of phosphorylated eNOS^{Ser1177} (Figure 4F). These results suggest that chloroquine increases arterial NO production in SHR.

We hypothesized that the improved NO status in arteries from chloroquine-treated SHR may be due to diminished generation of reactive oxygen species (ROS). To test this hypothesis, MRA from adult rats were incubated with the SOD mimetic tempol prior to concentration-response curves to ACh. In MRA from WKY, incubation with tempol had no effect on the relaxation to ACh in either Veh- or chloroquine-treated groups (Figure 4G and 4H). In MRA from Veh-treated adult SHR, the contractile response to ACh was abolished after incubation with tempol (Figure 4I). However, there was no difference in the contractile response to ACh in MRA from chloroquine-treated adult SHR incubated with or without tempol (Figure 4J). Protein expression analysis revealed that chloroquine increased expression of Cu/Zn SOD (SOD1) in adult SHR MRA (Figure 4K), and plasma from chloroquine-treated adult SHR had lower lipid peroxidation product MDA, indicating a reduction in systemic oxidative stress (Figure 4L). Overall these results suggest that chloroquine improved NO bioavailability via a decrease in ROS generation.

Improved NO status after chloroquine treatment was also evident in SHR aorta. In aortic segments from WKY, incubation with L- NNA increased the sensitivity to NE in both Veh- and chloroquine-treated groups (Figure 5A and Figure 5B). Contraction to NE in aortic segments from Veh-treated adult SHR was not changed after NOS inhibition with L-NNA, indicating diminished NO (Figure 5C). However, contraction to NE in aortic segments from chloroquine-treated adult SHR was significantly increased after incubation with L-NNA (Figure 5D), indicating the presence of NO. As decreased NO in the aorta is a pre-cursor to aortic stiffening [25], we tested whether chloroquine could influence remodeling enzyme activity. Chloroquine decreased matrix metalloproteinase (MMP)2 activity in adult SHR aorta (Figure 5E). Therefore, these results demonstrate that chloroquine can improve conduit artery function, as well as resistance artery function in SHR, and this may prevent subsequent vascular remodeling.

4. DISCUSSION

The major findings of the present investigation were that chloroquine treatment lowers blood pressure in adult SHR, but does not prevent the development of high blood pressure in young SHR. Nevertheless, irrespective of SHR age, chloroquine improved endothelial function via inhibition of the COX enzymes, decreased ROS generation, improved NO bioavailability, and reduced MMP2 enzyme activity, all of which ameliorated the hypertensive vascular dysfunction (figure 6). Therefore, our findings in SHR further support the notion that anti-malarial agents can control hypertension independently of autoantibodies [15, 26] and glomerulonephritis [15, 27], which are symptomatic of patients with hypertension [28, 29] and autoimmune diseases [30, 31], respectfully.

Chloroquine and other antimalarial agents are frequently used as first line treatment options for patients with autoimmune diseases [11]. Not only do autoimmune patients present increased risk of developing cardiovascular diseases, partly attributable to hypertension and endothelial dysfunction [7, 8], but it has been rationalized that elements of an autoimmune response were associated with the development of hypertension [3, 4]. However, whether chloroquine can reverse or prevent high blood pressure and vascular dysfunction in a model of essential hypertension was not previously known. Indeed, our results are in agreement with studies using other immunosuppressive strategies, including mycophenolate mofetil (MMF) that depletes T and B cells [32] and thymectomy [33], which has been reported to reduce hypertension in SHR.

In accordance with our lowered blood pressure in adult SHR, toxicity studies with chloroquine have observed a reduction in blood pressure in humans and rats [34, 35], as well as the magnitude of pressor responses to cold immersion in humans [34]. However, the mechanisms underlying these reductions were not elucidated from these studies. With respect to our data, we do not understand why chloroquine-treatment was only able to decrease blood pressure in adult SHR, but not prevent the age-dependent increase in blood pressure in young SHR. We can infer that there are functional and structural changes in the vasculature that chloroquine is able to reverse, but not prevent, and this can be the reason why chloroquine had different effects on blood pressure between adult and young SHR. Knowledge of this age-dependent efficacy of chloroquine to lower blood pressure may be

important clinically when considering treatment options for an adult or a child suffering with an autoimmune disease.

Another major finding of our investigation was that chloroquine abolished the contribution of COX enzymes to endothelial dysfunction in SHR. COX-derived prostaglandins and stimulation of thromboxane-prostanoid (TP) receptors have been well established as key mediators of endothelial inflammatory responses in hypertension [36, 37]. In the vasculature, COX 1 is expressed constitutively, but can also be overexpressed (e.g., by shear stress [38]), while COX 2 is generally considered inducible via nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) at sites of inflammation [39, 40]. However, COX 2 can be also expressed constitutively in several organs and cell types, including the endothelium, where its expression is also upregulated by shear stress [41]. In MRA from young SHR, we observed that chloroquine decreased expression of COX 1 and COX 2, and prevented ACh-induced contraction. In MRA from adult SHR, changes in expression of either COX enzyme were not evident, even though contraction to ACh was abolished and there was a reduced production of thromboxane B₂ (stable metabolite of Thromboxane A₂), signifying decreased activity. Despite these age-related differences in expression of COX enzymes in SHR MRA, our data suggest that chloroquine decreased the formation of COX-derived prostanoids (e.g., thromboxane A₂), which are known to significantly contribute to the pro-inflammatory milieu in hypertension [39] and autoimmune diseases [42].

Interestingly, our results indicated that chloroquine had no effect on the production of 6-keto prostaglandin F_{1 α} (stable metabolite of prostacyclin). Prostacyclin is generally considered a vasodilator in the vasculature, as well as an inhibitor of platelet aggregation. However in SHR, prostacyclin can also serve as an endothelium-derived contracting factor (EDCF) [43] and excess ROS production can cause inactivation of prostacyclin synthase and promote activation of TP receptors via redirection of arachidonic acid metabolism [44, 45]. Our results suggest that prostacyclin is not acting as contracting factor in our SHR, as 6-keto prostaglandin F_{1 α} was reduced in SHR compared to WKY, and chloroquine-treatment did not reconstitute expression to normotensive levels, despite the contractions to ACh being abolished. Furthermore, our biomarkers of ROS generation suggested that chloroquine-treatment reduced oxidative stress, which should preserve prostacyclin synthase activity, and decrease TP receptor-dependent contractions [44, 45]. However, our measurement of 6-keto prostaglandin F_{1 α} did not suggest that preservation of prostacyclin synthase activity was contributing to the reduced contractions to ACh. Therefore, our results suggest that chloroquine decreases ubiquitous COX activity, and not the specific activity of any of its downstream enzymes (e.g., prostacyclin synthase or thromboxane synthase).

As mentioned above, another important finding in our investigation was that chloroquine abolished the contribution of ROS to ACh-induced contractions. In fact, the concentration-response curves to ACh in the presence of tempol mimicked the concentration response curves to ACh in the presence of indomethacin. As the COX enzymes are known to be a prominent source of ROS in the vasculature [46] and ROS can act as amplifiers of EDCF-mediated responses [47], our data imply that chloroquine might not only lower the production of COX-derived ROS but also suppress participation of ROS to EDCF-mediated responses. Moreover, we observed that chloroquine was able to increase expression of the

antioxidant enzyme Cu/Zn SOD and decrease plasma lipid peroxidation, indicating heightened local antioxidant defense in the vasculature and a reduction in systemic oxidative stress respectively. Thus, decreased ROS and increased Cu/Zn SOD would improve the bioavailability of NO [48, 49]. It is well known that NO generation is required for endothelium-dependent vasodilatation and therefore, the NO-ROS balance plays an important mechanistic role in mediating endothelial (dys)function, and in the case of autoimmune diseases, this occurs even before the onset of proteinuria and increases in blood pressure [15, 50]. Interestingly, the improvements in endothelial function we observed after chloroquine treatment in SHR could be attributed to improvements in both eNOS activity (even though there were no differences in basal phospho-eNOS^{Ser1177} expression) and decreases in ROS. Overall, our results are very much in agreement with others who have reported that chloroquine and hydroxychloroquine are able to improve endothelium relaxation via increased NO bioavailability and decreased ROS in arteries from healthy [51] and diseased [15] rodents. Nonetheless, the underlying mechanisms for these improvements in endothelial function have been attributed to other sources of ROS, including NADPH-oxidase [15], and stimulation of NOS by chloroquine directly [51].

In this study we examined both resistance arteries (MRA) and conduit arteries (aorta) because both regions of the arterial tree have important functions on the propagation of blood flow through the circulatory system. While it is well established that small arteries and arterioles directly contribute to vascular resistance (and thus blood pressure), compliant conduit arteries provide important buffering actions for each ventricular contraction that alleviate pulse pressure [52]. When conduit artery stiffening occurs, pulsatile hemodynamics becomes augmented and this leads to increased cardiac afterload, decreased diastolic blood flow to tissues, detrimental vascular remodeling of resistance arteries, and end organ damage [52, 53]. After chloroquine-treatment in SHR, not only was resistance artery function improved, but we also report improvements in conduit artery function. Specifically, we observed improved NO status and decreased activity of MMP2 in SHR aorta after chloroquine treatment. Matrix metalloproteinase enzymes are involved in restructuring of arteries and the surrounding matrix by degrading, as well as stimulating matrix deposition [54]. Therefore, decreased MMP2 activity could subsequently diminish the progression of aortic stiffening, which is known to precede the development of high blood pressure [55]. These results support the observations that hydroxychloroquine treatment increases arterial elasticity, indicative of vascular compliance, in patients with SLE [26].

In conclusion, our results reveal several novel anti-hypertensive mechanisms of chloroquine in the vasculature that include inhibition of the COX enzymes, reduction of ROS generation, improved NO bioavailability, and decreased MMP activity. In turn, these mechanisms may lower the risk of cardiovascular disease in patients with autoimmune disease complicated with hypertension [7, 8]. These vasculature-specific anti-hypertensive actions of chloroquine may also reveal why anti-malarial treatments are protective against thrombovascular events [56] and decrease the presence of atherosclerosis [57, 58] in patients with autoimmune diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Dharmashankar K, Widlansky ME. Vascular endothelial function and hypertension: Insights and directions. *Curr Hypertens Rep.* 2010 Dec; 12(6):448–55. [PubMed: 20857237]
2. Okuda T, Grollman A. Passive transfer of autoimmune induced hypertension in the rat by lymph node cells. *Tex Rep Biol Med.* 1967 Summer;25(2):257–64. [PubMed: 6040652]
3. Mathis KW, Broome HJ, Ryan MJ. Autoimmunity: An underlying factor in the pathogenesis of hypertension. *Curr Hypertens Rep.* 2014 Apr.16(4) 424,014-0424-1.
4. Rodriguez-Iturbe B, Pons H, Quiroz Y, Lanaspa MA, Johnson RJ. Autoimmunity in the pathogenesis of hypertension. *Nat Rev Nephrol.* 2014 Jan; 10(1):56–62. [PubMed: 24247285]
5. McCarthy CG, Gouloupoulou S, Wenceslau CF, Spitler K, Matsumoto T, Webb RC. Toll-like receptors and damage-associated molecular patterns: Novel links between inflammation and hypertension. *Am J Physiol Heart Circ Physiol.* 2014 Jan 15; 306(2):H184–96. [PubMed: 24163075]
6. Chan CT, Sobey CG, Lieu M, Ferens D, Kett MM, Diep H, et al. Obligatory role for B cells in the development of angiotensin II-dependent hypertension. *Hypertension.* 2015 Nov; 66(5):1023–33. [PubMed: 26351030]
7. Aranow C, Ginzler EM. Epidemiology of cardiovascular disease in systemic lupus erythematosus. *Lupus.* 2000; 9(3):166–9. [PubMed: 10805482]
8. Mellana WM, Aronow WS, Palaniswamy C, Khera S. Rheumatoid arthritis: Cardiovascular manifestations, pathogenesis, and therapy. *Curr Pharm Des.* 2012; 18(11):1450–6. [PubMed: 22364129]
9. Scherbel AL, Schuchter SL, Harrison JW. Comparison of effects of two antimalarial agents, hydroxychloroquine sulfate and chloroquine phosphate, in patients with rheumatoid arthritis. *Cleve Clin Q.* 1957 Apr; 24(2):98–104. [PubMed: 13427154]
10. Tye MJ, White H, Appel B, Ansell HB. Lupus erythematosus treated with a combination of quinacrine, hydroxychloroquine and chloroquine. *N Engl J Med.* 1959 Jan 8; 260(2):63–6. [PubMed: 13613503]
11. Croyle L, Morand EF. Optimizing the use of existing therapies in lupus. *Int J Rheum Dis.* 2015 Feb; 18(2):129–37. [PubMed: 25884457]
12. Wozniacka A, Carter A, McCauliffe DP. Antimalarials in cutaneous lupus erythematosus: Mechanisms of therapeutic benefit. *Lupus.* 2002; 11(2):71–81. [PubMed: 11958581]
13. Wallace DJ. Does hydroxychloroquine sulfate prevent clot formation in systemic lupus erythematosus? *Arthritis Rheum.* 1987 Dec; 30(12):1435–6.
14. Petri M. Thrombosis and systemic lupus erythematosus: The hopkins lupus cohort perspective. *Scand J Rheumatol.* 1996; 25(4):191–3. [PubMed: 8792794]
15. Gomez-Guzman M, Jimenez R, Romero M, Sanchez M, Zarzuelo MJ, Gomez-Morales M, et al. Chronic hydroxychloroquine improves endothelial dysfunction and protects kidney in a mouse model of systemic lupus erythematosus. *Hypertension.* 2014 Aug; 64(2):330–7. [PubMed: 24842914]
16. Long L, Yang X, Southwood M, Lu J, Marciniak SJ, Dunmore BJ, et al. Chloroquine prevents progression of experimental pulmonary hypertension via inhibition of autophagy and lysosomal bone morphogenetic protein type II receptor degradation. *Circ Res.* 2013 Apr 12; 112(8):1159–70. [PubMed: 23446737]

17. Mulvany MJ, Halpern W. Mechanical properties of vascular smooth muscle cells in situ. *Nature*. 1976 Apr 15; 260(5552):617–9. [PubMed: 1264225]
18. McCarthy CG, Wenceslau CF, Gouloupoulou S, Ogbi S, Baban B, Sullivan JC, et al. Circulating mitochondrial DNA and toll-like receptor 9 are associated with vascular dysfunction in spontaneously hypertensive rats. *Cardiovasc Res*. 2015 Jul 1; 107(1):119–30. [PubMed: 25910936]
19. Catella F, Healy D, Lawson JA, FitzGerald GA. 11-dehydrothromboxane B2: A quantitative index of thromboxane A2 formation in the human circulation. *Proc Natl Acad Sci U S A*. 1986 Aug; 83(16):5861–5. [PubMed: 3461463]
20. Rosenkranz B, Fischer C, Reimann I, Weimer KE, Beck G, FRolich JC. Identification of the major metabolite of prostacyclin and 6-ketoprostaglandin F1 alpha in man. *Biochim Biophys Acta*. 1980 Aug 11; 619(2):207–13. [PubMed: 6996737]
21. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Anal Biochem*. 1982 Oct; 126(1):131–8. [PubMed: 7181105]
22. Spitler KM, Webb RC. Endoplasmic reticulum stress contributes to aortic stiffening via proapoptotic and fibrotic signaling mechanisms. *Hypertension*. 2014 Mar; 63(3):e40–5. [PubMed: 24379182]
23. Elgebaly MM, Prakash R, Li W, Ogbi S, Johnson MH, Mezzetti EM, et al. Vascular protection in diabetic stroke: Role of matrix metalloprotease-dependent vascular remodeling. *J Cereb Blood Flow Metab*. 2010 Dec; 30(12):1928–38. [PubMed: 20664613]
24. Luscher TF, Vanhoutte PM. Endothelium-dependent contractions to acetylcholine in the aorta of the spontaneously hypertensive rat. *Hypertension*. 1986 Apr; 8(4):344–8. [PubMed: 2870025]
25. Isabelle M, Simonet S, Ragonnet C, Sansilvestri-Morel P, Clavreul N, Vayssettes-Courchay C, et al. Chronic reduction of nitric oxide level in adult spontaneously hypertensive rats induces aortic stiffness similar to old spontaneously hypertensive rats. *J Vasc Res*. 2012; 49(4):309–18. [PubMed: 22572574]
26. 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 Jun. 1108:24–34. [PubMed: 17893967]
27. Petrin J, Rozman B, Dolenc P, Logar D, Bozic B, Vizjak A, et al. The dissociation of arterial hypertension and lupus glomerulonephritis in systemic lupus erythematosus. *Blood Press*. 1993 Jun; 2(2):108–12. [PubMed: 8180722]
28. Ofosu-Appiah W, Huang LY, Kuhnle M, Sfeir G, Kennel A. Autoantibodies against arterial antigens: Characterization by ELISA and immunoblot analysis in the spontaneously hypertensive rat. *Clin Exp Hypertens*. 1996 Jan; 18(1):21–35. [PubMed: 8822231]
29. Wenzel K, Haase H, Wallukat G, Derer W, Bartel S, Homuth V, et al. Potential relevance of alpha(1)-adrenergic receptor autoantibodies in refractory hypertension. *PLoS One*. 2008; 3(11):e3742. [PubMed: 19011682]
30. Budman DR, Steinberg AD. Hypertension and renal disease in systemic lupus erythematosus. *Arch Intern Med*. 1976 Sep; 136(9):1003–7. [PubMed: 962443]
31. Swaak AJ, van den Brink HG, Smeenk RJ, Manger K, Kalden JR, Tosi S, et al. Systemic lupus erythematosus: Clinical features in patients with a disease duration of over 10 years, first evaluation. *Rheumatology (Oxford)*. 1999 Oct; 38(10):953–8. [PubMed: 10534545]
32. Rodriguez-Iturbe B, Quiroz Y, Nava M, Bonet L, Chavez M, Herrera-Acosta J, et al. Reduction of renal immune cell infiltration results in blood pressure control in genetically hypertensive rats. *Am J Physiol Renal Physiol*. 2002 Feb; 282(2):F191–201. [PubMed: 11788432]
33. Khraibi AA, Smith TL, Hutchins PM, Lynch CD, Dusseau JW. Thymectomy delays the development of hypertension in okamoto spontaneously hypertensive rats. *J Hypertens*. 1987 Oct; 5(5):537–41. [PubMed: 3429859]
34. Anigbogu CN, Adigun SA, Inyang I, Adegunloye BJ. Chloroquine reduces blood pressure and forearm vascular resistance and increases forearm blood flow in healthy young adults. *Clin Physiol*. 1993 Mar; 13(2):209–16. [PubMed: 8453873]
35. Sofola OA, Olude IO, Adegoke F. The effect of chronic chloroquine toxicity on blood pressure of rats. *J Trop Med Hyg*. 1981 Dec; 84(6):249–52. [PubMed: 7321072]

36. Ricciotti E, FitzGerald GA. Prostaglandins and inflammation. *Arterioscler Thromb Vasc Biol.* 2011 May; 31(5):986–1000. [PubMed: 21508345]
37. Feletou M, Huang Y, Vanhoutte PM. Vasoconstrictor prostanoids. *Pflugers Arch.* 2010 May; 459(6):941–50. [PubMed: 20333529]
38. Doroudi R, Gan LM, Selin Sjogren L, Jern S. Effects of shear stress on eicosanoid gene expression and metabolite production in vascular endothelium as studied in a novel biomechanical perfusion model. *Biochem Biophys Res Commun.* 2000 Mar 5; 269(1):257–64. [PubMed: 10694510]
39. Feletou M, Huang Y, Vanhoutte PM. Endothelium-mediated control of vascular tone: COX-1 and COX-2 products. *Br J Pharmacol.* 2011 Oct; 164(3):894–912. [PubMed: 21323907]
40. Appleby SB, Ristimaki A, Neilson K, Narko K, Hla T. Structure of the human cyclo-oxygenase-2 gene. *Biochem J.* 1994 Sep 15; 302(Pt 3):723–7. [PubMed: 7945196]
41. Funk CD, FitzGerald GA. COX-2 inhibitors and cardiovascular risk. *J Cardiovasc Pharmacol.* 2007 Nov; 50(5):470–9. [PubMed: 18030055]
42. Rocca B, FitzGerald GA. Cyclooxygenases and prostaglandins: Shaping up the immune response. *Int Immunopharmacol.* 2002 Apr; 2(5):603–30. [PubMed: 12013502]
43. Gluais P, Lonchampt M, Morrow JD, Vanhoutte PM, Feletou M. Acetylcholine-induced endothelium-dependent contractions in the SHR aorta: The janus face of prostacyclin. *Br J Pharmacol.* 2005 Nov; 146(6):834–45. [PubMed: 16158068]
44. Zou MH, Leist M, Ullrich V. Selective nitration of prostacyclin synthase and defective vasorelaxation in atherosclerotic bovine coronary arteries. *Am J Pathol.* 1999 May; 154(5):1359–65. [PubMed: 10329589]
45. Zou MH, Shi C, Cohen RA. High glucose via peroxynitrite causes tyrosine nitration and inactivation of prostacyclin synthase that is associated with thromboxane/prostaglandin H(2) receptor-mediated apoptosis and adhesion molecule expression in cultured human aortic endothelial cells. *Diabetes.* 2002 Jan; 51(1):198–203. [PubMed: 11756341]
46. Virdis A, Bacca A, Colucci R, Duranti E, Fornai M, Materazzi G, et al. Endothelial dysfunction in small arteries of essential hypertensive patients: Role of cyclooxygenase-2 in oxidative stress generation. *Hypertension.* 2013 Aug; 62(2):337–44. [PubMed: 23734008]
47. Matsumoto T, Gouloupoulou S, Taguchi K, Tostes RC, Kobayashi T. Constrictor prostanoids and uridine adenosine tetraphosphate: Vascular mediators and therapeutic targets in hypertension and diabetes. *Br J Pharmacol.* 2015 Aug; 172(16):3980–4001. [PubMed: 26031319]
48. Grunfeld S, Hamilton CA, Mesaros S, McClain SW, Dominiczak AF, Bohr DF, et al. Role of superoxide in the depressed nitric oxide production by the endothelium of genetically hypertensive rats. *Hypertension.* 1995 Dec; 26(6 Pt 1):854–7. [PubMed: 7490139]
49. Gryglewski RJ, Palmer RM, Moncada S. Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. *Nature.* 1986 Apr 3–9; 320(6061):454–6. [PubMed: 3007998]
50. 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 Feb; 292(2):R736–42. [PubMed: 16971374]
51. Pestana CR, Oishi JC, Salistre-Araujo HS, Rodrigues GJ. Inhibition of autophagy by chloroquine stimulates nitric oxide production and protects endothelial function during serum deprivation. *Cell Physiol Biochem.* 2015; 37(3):1168–77. [PubMed: 26402856]
52. Mitchell GF. Arterial stiffness and wave reflection: Biomarkers of cardiovascular risk. *Artery Res.* 2009 Jun 1; 3(2):56–64. [PubMed: 20161241]
53. Schiffrin EL. Vascular remodeling in hypertension: Mechanisms and treatment. *Hypertension.* 2012 Feb; 59(2):367–74. [PubMed: 22203749]
54. Olszynski K, Zimowska M. Structure and function of matrix metalloproteinases. *Postepy Biochem.* 2009; 55(1):76–84. [PubMed: 19514468]
55. Kaess BM, Rong J, Larson MG, Hamburg NM, Vita JA, Levy D, et al. Aortic stiffness, blood pressure progression, and incident hypertension. *JAMA.* 2012 Sep 5; 308(9):875–81. [PubMed: 22948697]
56. Pierangeli SS, Harris EN. In vivo models of thrombosis for the antiphospholipid syndrome. *Lupus.* 1996 Oct; 5(5):451–5. [PubMed: 8902780]

57. Roman MJ, Shanker BA, Davis A, Lockshin MD, Sammaritano L, Simantov R, et al. Prevalence and correlates of accelerated atherosclerosis in systemic lupus erythematosus. *N Engl J Med*. 2003 Dec 18; 349(25):2399–406. [PubMed: 14681505]
58. Ravenell RL, Kamen DL, Spence JD, Hollis BW, Fleury TJ, Janech MG, et al. Premature atherosclerosis is associated with hypovitaminosis D and angiotensin-converting enzyme inhibitor non-use in lupus patients. *Am J Med Sci*. 2012 Oct; 344(4):268–73. [PubMed: 22222338]

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Figure 1. Chloroquine (CQ) lowered blood pressure in adult SHR, but it did not impede the development of high blood pressure in young SHR. (A) Systolic blood pressure (Post-SBP) and (B) heart rate (Post-HR) after 21 days of Veh or CQ treatment (40 mg/kg/day via intraperitoneal injection) in WKY, young SHR, and adult SHR. $n=4-8$. Two-way ANOVA: * $p<0.05$ vs. adult SHR-Veh.

Figure 2. Chloroquine (CQ) abolished the contractile response to acetylcholine (ACh) in mesenteric resistance arteries (MRA) of SHR. Concentration-response curves to ACh and sodium-nitroprusside (SNP) in MRA from Veh- or CQ-treated (A and B) WKY, (C and D) adult SHR, and (E and F) young SHR. n=4–6. Two-way ANOVA: *p<0.05 vs. Veh.

Figure 3.

Chloroquine (CQ) abolished the contractile response to acetylcholine (ACh) in SHR mesenteric resistance arteries (MRA) via cyclooxygenase (COX) inhibition. Concentration-response curves to acetylcholine (ACh), with or without indomethacin (10^{-5} mol/L), in MRA from Veh- or CQ-treated (A and B) WKY, (E and F) adult SHR, and (K and L) young SHR. Protein expression analysis for COX 1 and COX 2, both normalized for β actin, in (C and D) WKY, (G and H) adult SHR, and (M and N) young SHR MRA. *Above*, representative images of immunoblots. *Below*, densitometric analysis. (I) Thromboxane B₂ (TXB₂) measurement from MRA incubated in physiological salt solution. (J) 6-keto Prostaglandin F_{1α} (6-keto PGF_{1α}) measurement from plasma. $n=3-7$. Two-way ANOVA and Student's t-test: * $p<0.05$ vs. Veh. One-way ANOVA: * $p<0.05$ vs. WKY-Veh; # $p<0.05$ vs WKY-CQ; † $p<0.05$ vs. SHR-Veh.

Figure 4.

Chloroquine (CQ) increases nitric oxide production and bioavailability via decreased reactive oxygen species in SHR mesenteric resistance arteries (MRA). Concentration-response curves to acetylcholine (ACh), with or without L-NNA (10^{-4} mol/L), in MRA from Veh- or CQ-treated (A and B) WKY and (C and D) adult SHR. (E) Nitrite (NO₂⁻) and nitrate (NO₃⁻) measurement from MRA of adult SHR incubated in physiological salt solution. Protein expression analysis: (F) Phosphorylated eNOS^{Ser1177} normalized for total eNOS in adult SHR MRA. *Above*, representative images of immunoblots. *Below*, densitometric analysis. Concentration-response curves to ACh, with or without tempol (10^{-3} mol/L), in MRA from Veh- or CQ-treated (G and H) WKY and (I and J) adult SHR. Protein expression analysis: (K) Cu/Zn SOD normalized for β actin in adult SHR MRA. *Above*, representative images of immunoblots. *Below*, densitometric analysis. (L) Plasma measurement of the lipid peroxidation product malondialdehyde (MDA). (M and N) Concentration-response curves to ACh, with or without L-NNA (10^{-4} mol/L), in MRA from Veh- or CQ-treated young SHR. n=4–8. Log EC₅₀, Two-way ANOVA, and Student's t-test: *p<0.05 vs. Veh; †p<0.05 vs. CQ. One-way ANOVA: *p<0.05 vs. WKY-Veh; †p<0.05 vs. SHR-Veh.

Figure 5. Chloroquine (CQ) maintained nitric oxide and decreased the activity of remodeling enzyme matrix metalloproteinase (MMP)2 in SHR aorta. Concentration-response curves to norepinephrine, with or without L-NNA (10^{-4} mol/L), in aortic segments from Veh- or CQ-treated (A and B) WKY and (C and D) adult SHR. (E) Gelatin zymography of MMP2 activity. *Above*, representative images. *Below*, densitometric analysis. $n=4-6$. LogEC₅₀ or E_{max}: * $p<0.05$ vs. Veh; † $p<0.05$ vs. CQ. One-way ANOVA: * $p<0.05$ vs. WKY-Veh; † $p<0.05$ vs. SHR-Veh.

Figure 6. Schematic summarizing the anti-hypertensive effects chloroquine in the vasculature, and particularly endothelial cells. Chloroquine is a lysomotropic agent that raises the pH of endosomes (due to its properties as a weak base). Impaired endosomal acidification in the vasculature inhibits cyclooxygenase (COX) enzymes, reduces the generation of reactive oxygen species, improves nitric oxide (NO) bioavailability, and decreases the activity of matrix metalloproteinases (MMP) enzymes. Collectively, these mechanisms reduce peripheral resistance and ameliorate the pro-inflammatory phenotype in SHR.

Table 1

Chloroquine (CQ) increases 24 h urinary sodium concentration.

	WKY-Veh	WKY-CQ	SHR-Veh	SHR-CQ
Food consumption (g/day)	19.2±0.9	17.7±1.3	19.6±1.4	25.2±2.2 *
Water consumption (ml/day)	29.3±2.0	27.9±1.8	33.3±2.3	33.0±2.5
Urine volume (ml/day)	7.1±0.8	10.4±0.9	13.0±2.4	13.9±2.7
Urinary sodium excretion (mmol/day)	0.9±0.1	1.2±0.1	1.0±0.1	1.6±0.1 * [†]

n=3–5.

One-way ANOVA:

* p<0.05 vs. WKY-CQ;

[†] p<0.05 vs. WKY-Veh and SHR-Veh.