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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.

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

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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 libitium*. 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% O_2 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 μg) 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 A_2, thromboxane B_2 was measured as an estimate of *in vivo* thromboxane A_2 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 B_2 was then measured in the supernatant using a competitive ELISA kit and according to