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Anti-hypertensive effects of Rosuvastatin are associated with decreased inflammation and oxidative stress markers in hypertensive rats

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Abstract

Among their pleiotropic effects, statins exert antioxidant and anti-inflammatory properties. The aim of this study was to evaluate in normotensive (WKY) and in spontaneously hypertensive rats (SHR) the effect of rosuvastatin (ROSU) treatment on (1) plasma inflammation markers and endogenous NO synthase inhibitor (ADMA) levels, (2) reactive oxygen species (ROS) generated by circulating leukocytes and (3) vascular oxidative stress and tissue inflammation markers. Plasma cytokines were higher in SHR than in WKY, except for IL-4, which was lower in SHR than in WKY. SHR monocytes exhibited higher production of ROS than did WKY monocytes. In the experimental conditions, ROSU did not modify plasma cholesterol levels in SHR but attenuated the increase in systolic blood pressure. In SHR only, ROSU lessened pro-inflammatory cytokines and ADMA levels, increased IL-4 and reduced ROS production in circulating monocytes. These results demonstrate the beneficial effects of ROSU in SHR, independently of any lowering of cholesterol levels.

Keywords: *Statins, hypertension, cytokines, NAD(P)H oxidase, leukocytes*

Abbreviations: *ADMA, asymmetrical dimethyl-arginine; DAF-FM, 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate; DAPI, 4′,6-diamidino-2-phenyindole; DHE, dihydroethidium; EPA, endogenous peroxidase activity; GM-CSF, granulocyte macrophage colony-stimulating factor; HDL, high-density lipoprotein; HPLC, high performance liquid chromatography; HR, heart rate; IFN-γ, interferon-γ; IL, interleukin; iNOS, inductible NO synthase; LDL, low-density lipoprotein; MCP-1, monocyte chemoattractant protein-1; NAD(P)H, Nicotinamine Adenine Dinucleotide Phosphate; NO, nitric oxide;* $O_2^{\bullet -}$*, superoxide anion; PBS, phosphate-buffered saline; PMNs, polymorphonuclear cells; RLU, relative light units; ROS, reactive oxygen species; ROSU, rosuvastatin; SBP, systolic arterial pressure; SDMA, symmetric stereoisomer dimethyl-arginine; SHR, spontaneously hypertensive rats; WKY, Wistar Kyoto.*

Introduction

Hypertension is associated with vascular remodelling, endothelial dysfunction and contributes to the aggravation of ischemic heart disease [1,2]. There is increasing evidence that oxidative stress and inflammation are associated with the development of hypertension [3].

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Recently, an endogenous mechanism regulating nitric oxide synthesis has been described; this is nitric oxide synthase (NOS) inhibitors, including asymmetric dimethylarginine (ADMA), which can competitively inhibit NOS [4]. An increased level of ADMA plasma has been observed in many diseases such as hypertension, atherosclerosis and diabetes mellitus [4–6], suggesting that ADMA may be an important contributor to the development of endothelial dysfunction.

Indeed, over-production of superoxide anion ($O_2^{\bullet -}$) induces a reduction in NO bioavailability in vascular walls and increased peroxinitrite production [7]. Which, in turn, activates NFkB and induces the expression of chemokines in the endothelium [1,8]. Monocyte recruitment by monocyte chemoattractant protein-1 (MCP-1) is a major step in vascular wall inflammation [9]. A relationship between leukocyte-derived reactive oxygen species (ROS) and hypertension has been suggested [10]. A 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor (statin) limits the development of atherosclerosis and reduces cardiovascular mortality and morbidity. Independently of their action on blood lipids [11], statins reduce the *in vivo* induction of inflammatory mediators such as MCP-1, inhibit endothelial surface expression of cell adhesion molecules and decrease oxidative stress [12–15]. These observations support the hypothesis that statins are a novel means of attenuating oxidative stress and inflammatory processes associated with hypertension. Furthermore, we and others [16,17] have observed that the vascular effects of rosuvastatin (ROSU) could attenuate the increase of arterial blood pressure in spontaneously hypertensive rats (SHR). The aims of this study were to (1) determine inflammatory factors, leukocyte-related oxidative stress and asymmetrical dimethylarginine (ADMA) production in a genetically hypertensive rats (SHR) model as compared to a normotensive Wistar Kyoto strain (WKY) and (2) assess the pleiotropic effects of ROSU treatment in both strains of rats.

In this paper, we are able to show that treatment with rosuvastatin could attenuate the increased arterial blood pressure associated with a reduction of inflammatory and oxidative stress markers in spontaneously hypertensive rats, without modifying plasma cholesterol level.

Materials and methods

Animals

Procedures involving animals and their care were all conducted in conformity with the institutional guidelines, which comply with national and international laws and policies. Male adult (10-week old) SHR (200–220 g, $n = 20$) or WKY rats (200–230 g, $n = 20$), purchased from Charles River Laboratories (France), were used. They were housed in an animal room under temperature control ($24 \pm 0.5^{\circ}$C) and a 12-h light–dark (08:00–20:00) cycle. They received either vehicle or ROSU (10 mg/kg/day, Astrazeneca, France) for 3 weeks by gavage. Standard laboratory rat chows (Purina) and tap water were available *ad libitum*. The ROSU dose was chosen according to previous studies in rats [16–19]. All animals were allowed to acclimatize for at least 7 days prior to experimental manipulations.

Measurement of arterial pressure and heart rate

Systolic arterial pressure (SAP) and heart rate (HR) in conscious rats were measured every week using a non-invasive tail-cuff method (Bioseb, France). The rats were handled repeatedly and allowed to adapt to the restraint chamber for 3 days before the actual measurements. The mean of six readings was recorded as the individual SAP and HR.

Plasma cholesterol and ADMA levels

After the rats were killed, blood samples were collected from the abdominal aorta and centrifuged at 1500 rpm for 10 min. Plasma total cholesterol, high-density lipoprotein (HDL) and triglycerides were measured by colorimetric assay. Low-density lipoprotein (LDL) cholesterol was calculated with Friedwall methods. L-arginine, ADMA and its symmetric stereoisomer dimethyl-arginine (SDMA) were measured by high performance liquid chromatography (HPLC) as previously described [20]. Briefly, plasma (0.2 mL) was added to N-monomethyl L-arginine as the internal standard (0.1 mL, 25 μm) and phosphate-buffered saline (PBS) (0.7 mL). This mixture was applied to a cation-mixed mode polymeric sorbent (Phenomenex STRATA-X-C 33u, Le Pecq, France). The solid phase extraction cartridge was consecutively washed with HCl (100 mm) and methanol (1:1; v:v). The analytes were eluted with 1.0 mL of concentrated ammonia/water/methanol (10/40/50; v:v:v) by vacuum suction. The eluate, dried under nitrogen, was derivatised with *ortho*-phthaldialdehyde reagent (1:1; v:v) and injected into the HPLC system. The HPLC was equipped with a fluorescent detector (λ_{exc} 340 nm, λ_{em} 455 nm) and a LiChrospher®100 RP-18 column (250 × 4 mm, 5 μm) protected by a guard-column (4 × 4 mm, 5 μm) with the same stationary phase (Merck, Darmstadt, Germany). Analyte separation by HPLC was performed at room temperature. The detection limit was 0.1 μm. Assays were done in duplicate.

Endogenous peroxidase activity

A colorimetric test was used to quantify endogenous peroxidase activity (EPA) in plasma samples (LDN, Germany). Plasma samples were used according to

the manufacturer's procedure using an ELISA plate reader at 450 nm (Titertek Multiscan MCC/340, France). The results are expressed in peroxidase units (U/L).

Plasma cytokine levels

Interleukin (IL)-1α, IL-4, MCP-1, interferon-γ (IFN-γ) and granulocyte macrophage colony-stimulating factor (GM-CSF) concentrations were determined by Cytometric Bead Array (CBA; Bender MedSystems). Plasma was incubated with labelled capture beads and detection reagent for 3 h in the dark at room temperature and analysed with a flow cytometer (FACSCalibur; BD Biosciences) by using the respective CBA Analysis software (BD Biosciences) and Bender MedSystems software. Cytokine standards for quantification (pg/mL) as well as the blanks were handled in the same manner as the samples.

Leukocyte isolation and oxidative stress measurement

Production of ROS in peripheral blood was assessed by flow cytometry using fluorescent probes. Briefly, whole blood was diluted five times in PBS and incubated for 15 min at 37°C with either dihydroethidium (DHE, 10 μm, Invitrogen) for $O_2^{\bullet -}$ measurement or Mitosox (10 μm, Invitrogen) for $O_2^{\bullet -}$ produced by mitochondria or 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM, 10 μm, Invitrogen) for NO measurement. Blood cells incubated without fluorescent probes were used as controls. Cells were precipitated by centrifugation and the supernatant was discarded. The cell pellets were resuspended in 50 μL PBS. The red blood cells were then lysed by incubation for 10 min at room temperature with 2 mL of FACS lysing solution. The leukocytes were washed twice with PBS then resuspended and fixed in 500 μL of 1% paraformaldehyde. The suspension of fixed cells was used for flow cytometry. Flow cytometry was carried out by dual-colour analysing using a FACSCalibur (BD Biosciences, France) flow cytometer and analysed with WinMDI software (WinMDI 2.8, http://facs.scripps.edu/software.html). For each sample, data from 30 000 cells were collected and displayed in a dot-plot of forward-scatter and side-scatter. The cells were gated for lymphocytes, monocytes or polymorphonuclear cells (PMNs). Geometric mean of fluorescence intensity in the gated cell population was measured in FL1 channel for DAF-FM or in FL2 channel for DHE and Mitosox. Data were presented as geometric mean of fluorescence intensity after the background fluorescence intensity of controls had been subtracted [21].

Tissue processing

The thoracic aortas were excised and washed in 4°C 0.9% NaCl solution. Any adherent adipose tissue was carefully removed from the aortic segment. Two 5–10 mm-long rings were cut. One ring was embedded on OCT (Dako, France) and cut into 10 μm thickness sections which were stored at -80°C. One aorta segment was immediately frozen in liquid nitrogen and kept at -80°C until used for chemiluminescence.

Measurement of Nicotinamine Adenine Dinucleotide Phosphate (NAD(P)H) oxidase activity by lucigenin-enhanced chemiluminescence

The capacity of vascular tissue to produce $O_2^{\bullet -}$ in an NAD(P)H-dependent way was assessed using an LB 9507 luminometer (Berthold Systems, Aliquippa, PA) by the measurement of superoxide-enhanced lucigenin (0.5 μmol/L, Sigma) chemiluminescence in the presence or absence of NAD(P)H (30 μmol/L, Sigma). The results are expressed in relative light units (RLU) per gram of dry tissue. Briefly, the aortic tissue sections were thawed and pre-incubated in Krebs–HEPES buffer. Specificity for superoxide was confirmed by experiments in the presence of superoxide dismutase (SOD; 300 IU/mL; Sigma) [22].

In-situ *detection of superoxide anion*

DHE, an oxidative fluorescent probe, was used to localize $O_2^{\bullet -}$. The freshly-frozen thoracic aorta tissues were fixed for 10 min in acetone. Slides were incubated in a light-protected humidified chamber at room temperature with DHE (5 μmol/L) for 5 min. The slides were counterstained with a nuclear tracer (4′,6-diamidino-2-phenylindole (DAPI), Invitrogen 30 μg/mL). To verify the specific detection of $O_2^{\bullet -}$ by DHE, some slides were incubated with superoxide dismutase (300 U/mL, Dako) before DHE incubation (data not shown). The slides were immediately analysed with a computer-based digitizing image system (Microvision, Evry, France) using a fluorescent microscope (Eclipse 600, Nikon, Champigny-Sur-Marne, France) connected to a video camera (Tri CCD, Sony, Paris). Fluorescence was detected with 510–560 nm excitation and 590 nm emission filters. Automatic computer-based analysis was performed with the same threshold for all sections ($\times$ 500 magnification). Results are expressed as DHE fluorescence/DAPI ratio.

Localization of inductible NO synthase (INOS) and MCP-1 by histological methods

The freshly-frozen thoracic aorta tissues were fixed for 10 min in acetone and pre-incubated with 10% H_2O_2. Goat polyclonal antibodies (Santa Cruz, 1:100)

against directed MCP-1 and iNOS were applied and incubated for 15 h at 4°C. The sections were incubated with biotinylated anti-mouse rabbit immunoglobulins (Santa Cruz, 1:400) for 20 min and then with horseradish peroxidase-labelled streptavidin (Santa Cruz, 1:600) for 20 min. Peroxidase activity was revealed with aminoethylcarbazole (Dako). The slides were counterstained with haematoxylin (10–20 s) and mounted. To provide a quantitative measure of MCP-1 and iNOS staining, semi-quantitative scores were realized on 10 aortas slides of each groups. The classification of the relative staining into four scores at $\times 50$ magnification was 0 = absence; 1 = mild; 2 = moderate; 3 = marked as described previously [23].

Statistical analyses

All data are expressed as means ± SEM. Statistical analyses were performed with the two-factor analysis of variance (ANOVA) test (SigmaStat), the two factors being the type of rat (SHR vs WKY) and ROSU treatment. ANOVA was followed by inter-group pair-wise comparisons with Tukey HSD multiple comparisons.

Results

Physiological parameters

SAP and HR were measured *in vivo* throughout the treatment in WKY and SHR. At 10 weeks of age, the SAP of SHR was considerably higher, 40 mmHg, than in WKY (Table I) and the SAP progressively increased over the 3-week period ($p < 0.001$). The treatment of SHR with ROSU was able to stop the progression of hypertension in these rats ($p < 0.001$). For HR, there was no difference between WKY and SHR and it was not affected by ROSU treatment.

Plasma cholesterol and ADMA levels

Biochemical analysis of the plasma at the end of the treatment showed that SHR had lower levels of HDL, LDL and total cholesterol than WKY rats (data not shown). However, 3 weeks of daily treatment with ROSU did not modify cholesterol levels in either group of rats. There was no difference between strains and treatment groups for triglyceride levels (Table II).

Table I. Effect of ROSU (10 mg/kg/day for 3 weeks) on the evolution of systolic arterial pressure (mmHg) of WKY and SHR.

	WKY		SHR	
Rats treatment	Control	ROSU	Control	ROSU
Week 0	131 ± 3	131 ± 3	174 ± 2⋆	174 ± 2
Week 1	132 ± 2	134 ± 3	180 ± 3⋆	170 ± 2$
Week 2	132 ± 4	132 ± 2	190 ± 6⋆	176 ± 2$
Week 3	131 ± 1	130 ± 4	186 ± 2⋆	168 ± 2$

Results are presented as means ± SEM. ⋆$p < 0.001$ SHR control vs WKY control; $ $p < 0.001$ SHR control vs SHR + ROSU.

Table II. Plasma lipid levels of WKY and SHR rats after 3 weeks of water or ROSU treatment (10 mg/kg/day for 3 weeks) ($n = 10$ for each group).

	WKY		SHR	
Rats treatment	Control	ROSU	Control	ROSU
HDL (mg/dL)	39 ± 1	37 ± 1	27 ± 1⋆	28 ± 1§
LDL (mg/dL)	26 ± 2	24 ± 1	17 ± 2⋆	16 ± 1§
Total cholesterol (mg/dL)	70 ± 3	68 ± 2	52 ± 2⋆	50 ± 1§
Triglycerides (mg/dL)	44 ± 6	52 ± 7	49 ± 6	55 ± 5

Results are presented as means ± SEM. ⋆$p < 0.05$ SHR control vs WKY control; § $p < 0.05$ SHR + ROSU vs WKY + ROSU.

L-Arginine and ADMA concentrations were higher in SHR than WKY ($p < 0.05$) (Table III). However, no differences were observed between SHR and WKY for either SDMA concentrations or L-arginine/ADMA and ADMA/SDMA ratios. However, ROSU decreased ADMA plasma concentrations and increased the L-arginine/ADMA ratio in SHR.

No differences were observed in EPA activity whatever the animal strain or treatment (WKY vs SHR: 15.5 ± 5.5 vs 19.0 ± 4.5 U/L P = NS and WKY + ROSU vs SHR + ROSU 13.1 ± 3.2 vs 14.8 ± 4.5 U/L).

Plasma inflammatory marker levels

We detected and quantified different plasma cytokines such as IL-1α, IL-4, IFN-γ, GM-CSF, MCP-1 with a multiplex ELISA kit (Table III). IL-1α, IFN-γ, GM-CSF, MCP-1 levels were 30–50% higher in SHR than in WKY ($p < 0.05$). IL-4 levels were lower in SHR plasma than in WKY plasma ($p < 0.05$). ROSU treatment decreased IL-1α, IFN-γ, GM-CSF, MCP-1 and increased IL-4 in SHR ($p < 0.05$) but not in WKY.

Leukocyte oxidative stress status

DAF-FM, DHE and Mitosox geometric mean of florescence in lymphocytes, monocytes and PMNs was analysed in each of the four groups of rats. The geometric means of DHE, Mitosox and DAF-FM fluorescence were significantly higher in SHR monocytes than in WKY monocytes ($p < 0.05$) (Figure 1A–C). No differences were observed for lymphocytes and PMNs (data not shown). ROSU treatment decreased geometric mean of DHE, Mitosox and DAF-FM fluorescence in SHR monocytes ($p < 0.05$).

Oxidative stress and inflammatory status in rat aortas

Vascular NAD(P)H oxidase activity (Figure 2A) was higher in SHR than in WKY (6049 ± 90 vs 4530 ± 67 RLU/mg; $p < 0.05$). SOD in the medium reduced

Table III. Effect of ROSU (10 mg/kg/day for 3 weeks) on the cytokines plasma levels and nitric oxide metabolism in WKY and SHR.

	WKY		SHR	
Rats treatment	Control	ROSU	Control	ROSU
NO metabolism				
L-Arginine (μM)	69.0±13.3	69.4±15.3	111.5±12.5*	92.0±12.5
ADMA (μM)	1.1±0.3	1.2±0.4	2.3±0.3*	0.8±0.3$
SDMA (μM)	0.4±0.2	0.8±0.3	0.4±0.2	0.8±0.2
Ratio L-Arg/ADMA	77.4±12.0	63.8±14.7	69.8±12.0	109.7±12.0$
Ratio ADMA/SDMA	8.0±3.1	5.0±3.8	12.0±3.1	2.3±3.3
Inflammatory markers				
GM-CSF (pg/ml)	39.2±6.2	42.4±6.1	54.9±8.4*	29.4±5.5$
IL-1α (pg/ml)	80.3±12.2	88.3±13.3	125.0±11.0*	79.3±12.2$
IL-4 (pg/ml)	13.7±2.2	9.4±2.1	5.1±1.1*	10.0±2.0$
IFN-γ (pg/ml)	14.8±3.2	10.1±2.0	26.3±6.2*	11.8±3.4$
MCP-1 (pg/ml)	286.4±26.3	235.1±36.3	534.0±31.8*	453.4±33.8$

Results are presented as means±SEM. * $p<0.05$ SHR control vs WKY control; $, $p<0.05$ SHR control vs SHR+ROSU.

NADPH-dependent superoxide production by 90%. Three weeks of ROSU treatment significantly decreased vascular NAD(P)H oxidase activity in SHR aorta ($p<0.05$), this effect was not observed in WKY rats. In SHR aorta, DHE fluorescence (Figure 2B and C) was 25% higher than that in WKY (17.2±2.1 vs 11.3±1.1 emission mm^{-2}; $p<0.01$). The ROSU treatment decreased vascular DHE fluorescence by 30% in SHR ($p<0.01$) but not in WKY rat aorta.

MCP-1 (Figure 3A) and iNOS (Figure 3B) expression detected by immunohistochemistry in the intima and in the media were higher in SHR aorta than in WKY aorta ($p<0.01$). ROSU treatment reduced MCP-1 and iNOS expression in SHR aorta but not in WKY ($p<0.05$).

Discussion

The results of this study demonstrate that (1) the SHR model exhibits an increase in plasma inflammatory markers, ADMA levels associated with a higher degree of oxidative stress in aorta and in leukocytes, (2) ROSU treatment (10 mg/kg/day for 3 weeks) exerts independently of its actions on blood lipid levels beneficial effects in SHR; it lowers arterial blood pressure, the inflammatory index and ADMA levels concomitantly with a reduction in oxidative stress status in plasma, in circulating cells and in the aorta.

It has been documented that inflammation and oxidative stress play an important role in many cardiovascular diseases, including hypertension [24,25]. In our experimental model, 13-week-old SHR exhibit an SAP which is 40 mmHg higher than that in WKY. It has recently been confirmed that there is a common inflammatory basis for both endothelial dysfunction and hypertension [1]. The accumulation of plasma ADMA seems to be an early marker for inflammation and metabolic dysfunctions of blood vessels [26]. It has been shown that the level of ADMA, an endogenous inhibitor of NOS, is significantly increased in patients with essential hypertension [27]. In this way, ADMA has been reported to competitively inhibit NO synthesis by displacing L-arginine from NOS and studies in cultured human endothelial cells have shown that elevated ADMA results in the production of superoxide anion [28,29]. It is tempting to speculate that ADMA-induced eNOS uncoupling significantly contributes to superoxide anion production in endothelial cells. However, further studies are needed to assess the contribution of ADMA in eNOS uncoupling during hypertension.

ADMA is derived from the catabolism of proteins containing methylated arginine residues and is metabolized by an enzyme, dimethylarginine dimethylaminohydrolase (DDAH). Although DDAH plays an important role in the regulation of systemic ADMA levels [30], the regulatory mechanisms for DDAH expression in SHR have not been fully evaluated. However, Wakino et al. [31] showed that DDAH is expressed in SHR and WKY kidneys while no difference in the expression of this enzyme was noted between the two strains of rats.

In accordance with this new concept, we observed in our study an increase in ADMA plasma levels in SHR, indicating the aggravation of the inflammatory status of endothelial cells. The present data concerning inflammatory status, show increased plasma levels of MCP-1, GM-CSF, IFN-γ and IL-1α in SHR. These inflammation-related mediators correlated positively with the pathogenesis of vascular diseases [32]. The importance of inflammation in the latter stages of heart failure has been emphasized [33–35]. However, the roles of these cytokines in the development of hypertension are not well understood. Ishibashi et al. [36] suggested that MCP-1 receptor expression in monocytes plays a critical role in vascular inflammation and remodelling in angiotensin II-induced hypertension and possibly in other forms of hypertension. In our experimental conditions,

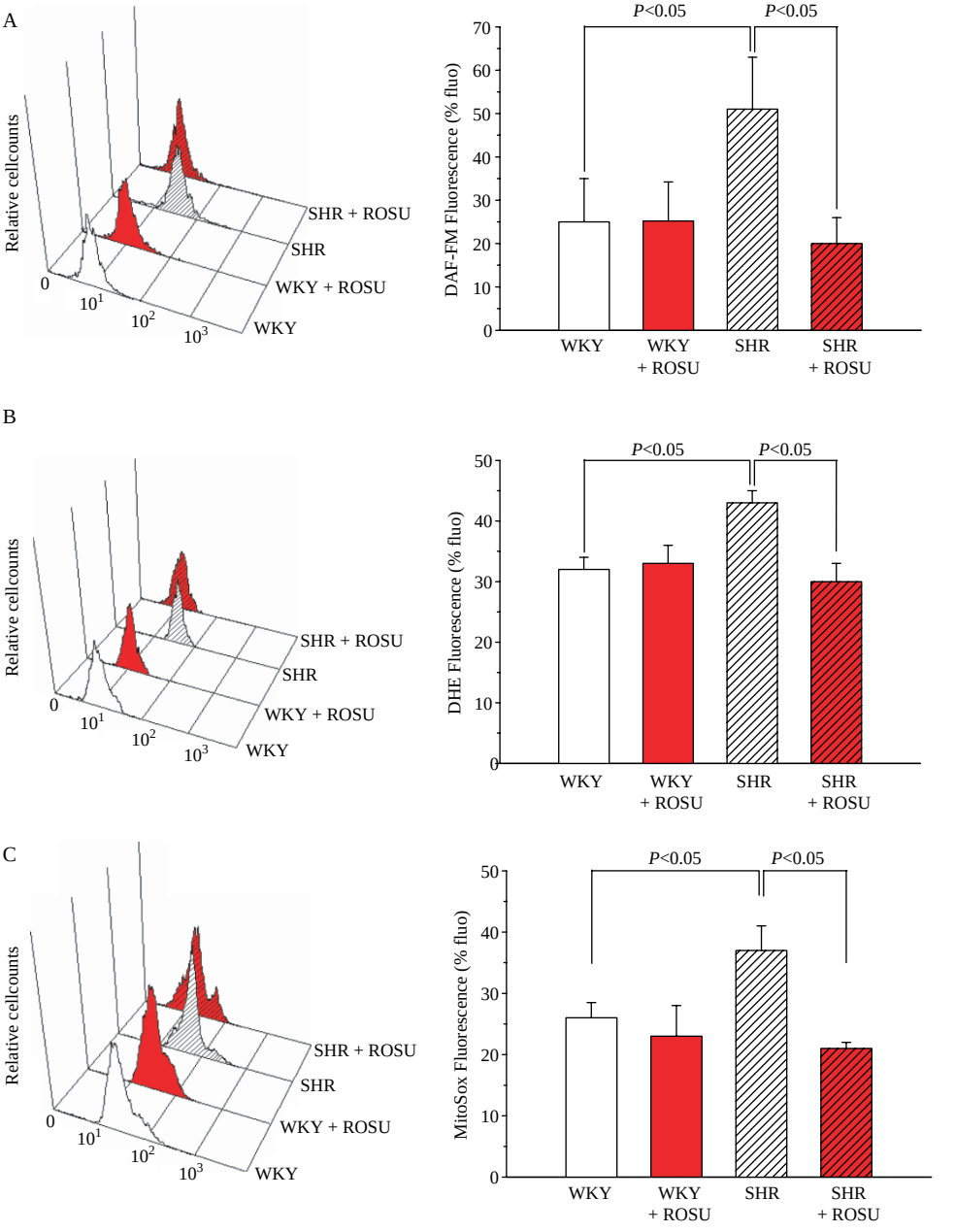

Figure 1. Fluorescence histograms of oxidative stress index measured by (A) Diaminofluorescein (DAF-FM), (B) dihydroethidium (DHE) and (C) MitoSox in peripheral blood monocytes of WKY and SHR treated or not with 10 mg/kg/day of ROSU for 3 weeks. Results are presented as means ± SEM.

hypertension in SHR was associated with an increase in MCP-1 plasma levels. Concerning GM-CSF and its importance in the pathogenesis and progression of arterial hypertension, Parissis et al. [37] found that patients with mild-to-moderate arterial hypertension had higher plasma concentrations of both MCP-1 and GM-CSF. Additionally, the levels of these inflammatory factors were higher in hypertensive patients with significant hyperlipidemia. It can be suggested that the elevation of serum cytokines may reflect the unfavourable effects of arterial pressure on endothelial function. It is important to observe that in our experimental conditions, MCP-1 expression was also detected in the intima and in the media and that this expression was higher in SHR aorta than in WKY aorta.

In our study, blood plasma levels of a Th1 cytokine, IFN-γ, were significantly higher in SHR than in WKY, whereas levels of a Th2 cytokine, IL-4, were significantly lower in SHR than in WKY. IL-4 and IFN-γ represent a well-known example of a pair of mutually counteracting cytokines. IL-4 drives the development of Th2 cells and thereby regulates antibody production or humoral immunity [38]. IL-4 exerts antioxidant properties including modulation of NO production through the inhibition of iNOS [39,40] and is able to decrease lipid peroxidation during ischemia-reperfusion sequences [41].

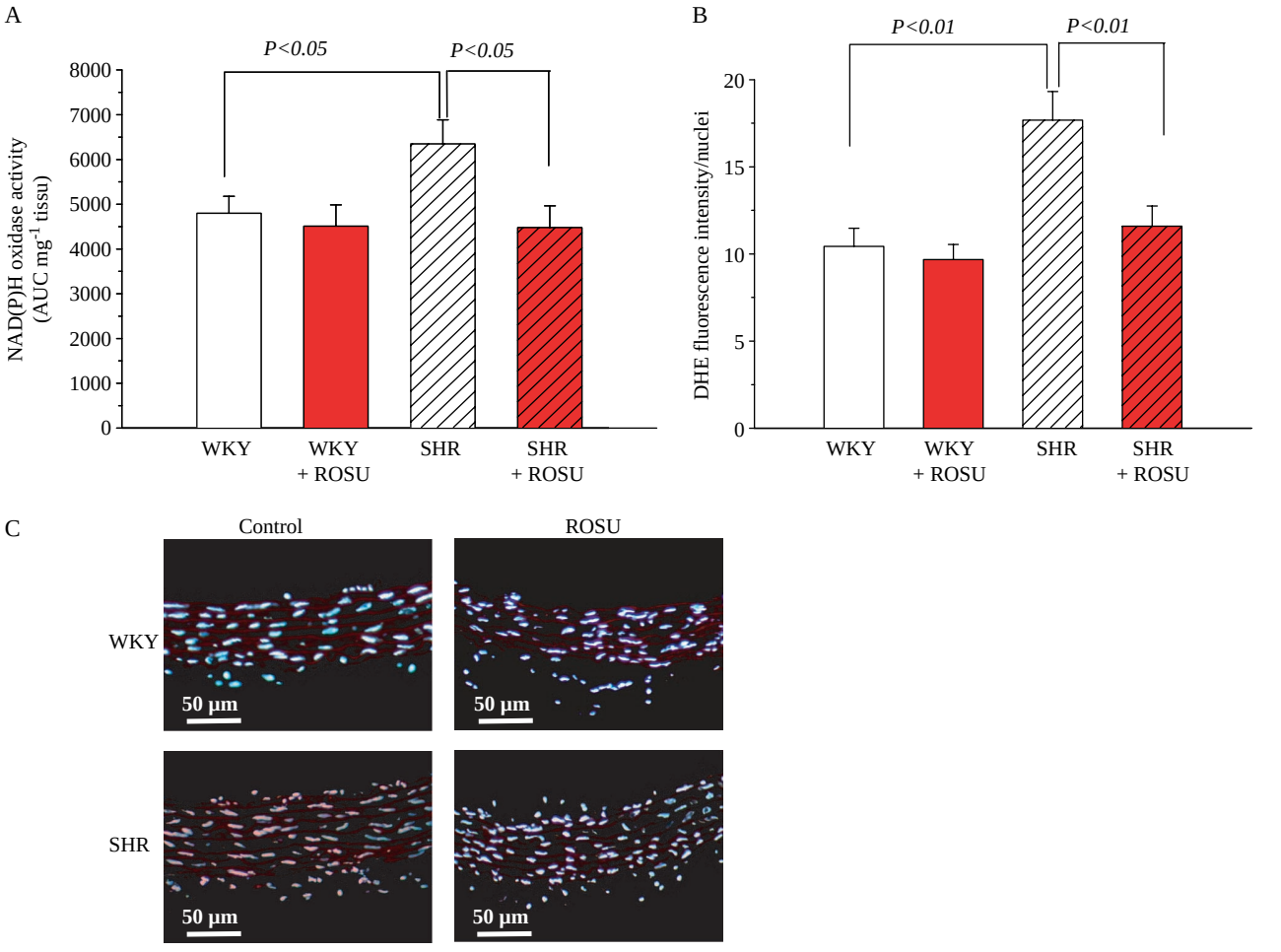

Figure 2. (A) Aorta NAD(P)H oxidase activity evaluated with lucigenin, (B) dihydroethidium (DHE) fluorescence intensity/nuclei and (C) typical DHE-DAPI double staining in WKY and SHR treated or not with 10 mg/kg/day of ROSU for 3 weeks. Results are presented as means ± SEM.

In contrast, IFN-γ stimulates cell-mediated immune response, including monocyte and macrophage production and promotes NO production by iNOS over-expression.

Data from several models of vascular injury have reported that angiotensin II was a major participant in the inflammatory process. The role of the renin-angiotensin system in the development of hypertension is well established in both human and animal models such as the SHR [42]. Dang et al. [43] reported that there were no significant differences in plasma angiotensin II levels between WKY and SHR strains from 5–20 weeks. However, left ventricular tissue and intra-renal angiotensin II concentrations were significantly higher in SHR than in WKY [44]. One action of angiotensin II that has received increasing attention is its ability to increase production of oxygen free radicals [45].

In our experimental conditions, our present study indicated that SHR monocytes produced higher levels of $O_2^{\cdot -}$ and NO than did WKY monocytes. We confirmed, in this study, that SHR display greater levels of vascular oxidative stress by the enhancement of NAD(P)H oxidase activity. Evidence indicates that membrane-bound NAD(P)H oxidases are the major source of free radical generation [46]. Among the multiple factors that are thought to play an essential role in the control of vascular tone, the balance between oxidative and antioxidant species is becoming increasingly significant. In this way, the role of NO as a modulator of arterial pressure associated with an inflammatory response is controversial. Numerous studies have demonstrated that NO synthesis may be high in SHR, probably as a counter-regulatory mechanism activated to compensate for the increase in blood pressure [47,48]. Our study demonstrated that iNOS expression in tissue was higher in SHR aorta than in WKY aorta. Under oxidative conditions excess generation of NO by iNOS contributes to the inflammatory response, through its reaction with $O_2^{\cdot -}$ to form a potent oxidant, peroxynitrite [7]. Recently, up-regulation of iNOS in aorta during the development of hypertension in SHR has been reported [49]. These findings underline the complexity of the relationship between oxidative stress, inflammation and the pathogenesis

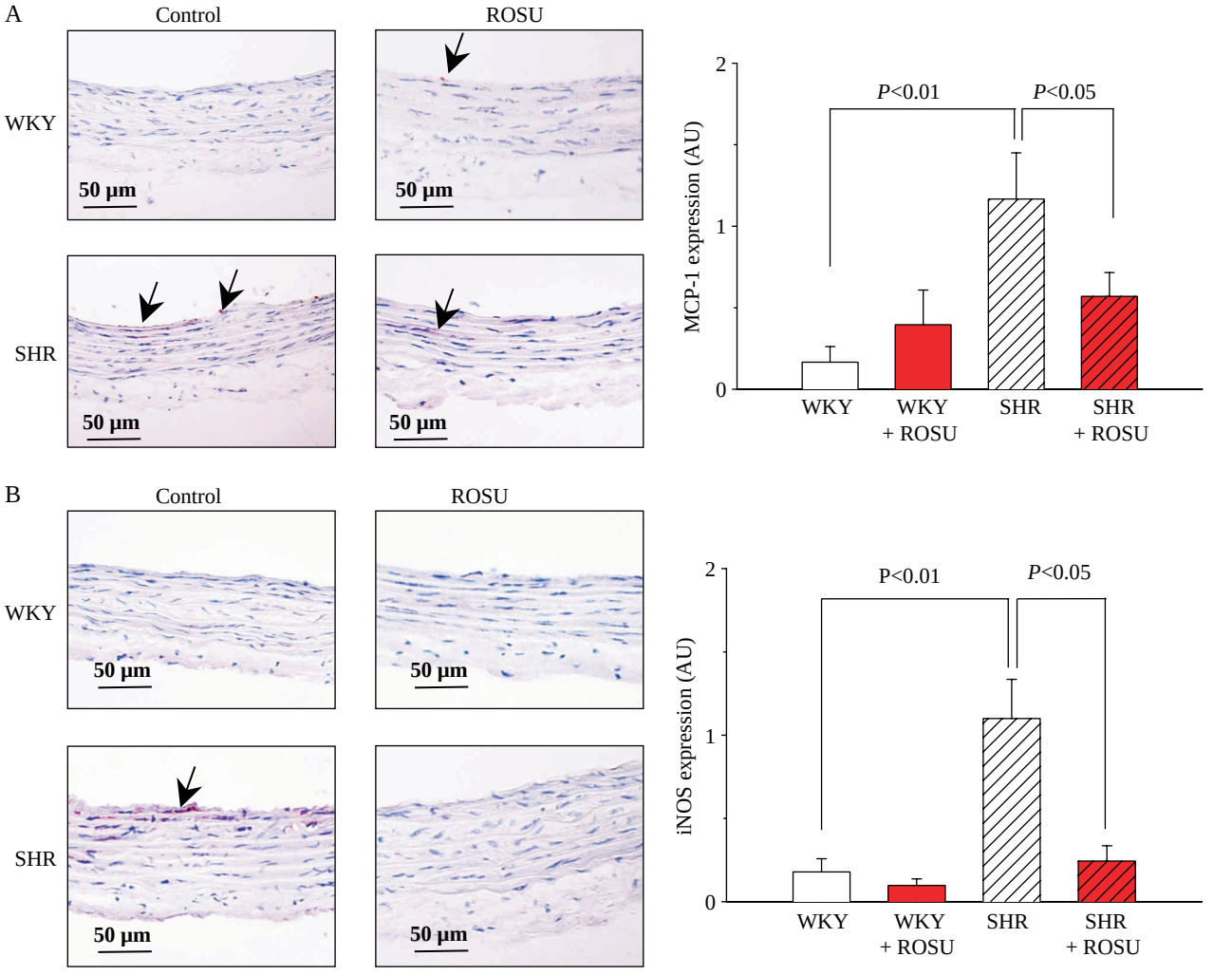

Figure 3. Immunohistochemical localization (arrows) and semi-quantification of vascular (A) monocyte chemoattractant protein-1 (MCP-1) and (B) inductible NO synthase (iNOS) expression in SHR and WKY aorta rat treated or not with 10 mg/kg/day of ROSU for 3 weeks. Results are presented as means ± SEM.

of hypertension. Several studies have demonstrated that the release of inflammation mediators in plasma was modulated by intracellular ROS production by leukocytes [50,51] and by the spontaneous activation and/or degranulation of circulating neutrophils and monocytes in both SHR and in humans with essential hypertension [52,53]. Moreover, it has been reported that the induction of hypertension in normotensive rats causes a spontaneous increase in ROS generation in the circulating leukocytes [21] leading to the inactivation of NO and the production of isoprostanes [54]. A substantial number of studies have shown increased generation of oxygen free radicals in association with reduced production of scavenging enzymes in hypertensive animal models [55]. Ito et al. [56] and a recent genetic study suggested that the transcription of diverse redox-regulatory genes may be modified in rodent models of human essential hypertension [57].

In our experimental conditions, concentrations of plasma HDL and LDL cholesterol in SHR groups were significantly lower than those in WKY groups; treatment with ROSU did not affect these plasma levels. These finding are consistent with other studies [17]. Independently of its actions on blood–lipid levels, ROSU attenuated the high SAP in SHR. The pressure-lowering effects of various statins have been reported previously in different hypertension models including SHR and in hypertensive patients [16,17,58]. Treatment with ROSU induced a decrease in ADMA plasma levels in SHR. This effect on the status of plasma ADMA accumulation is an interesting finding that suggests that ROSU induced an improvement in endothelial function. Yin and Xiong [59] found that pravastatin was able to restore DDAH activity in rat aorta and to enhance endothelium-dependent relaxation.

Our results showed that ROSU treatment reduced vascular oxidative stress in SHR but not in WKY aorta; this effect was associated with MCP-1 and iNOS expression and a reduction in NAD(P)H oxidase activity. Rosuvastatin was able to reduce

expression of NAD(P)H oxidase sub-units gp91phox, p40phox and p22phox in the heart [60].

Furthermore, ROSU lessened pro-inflammatory cytokine levels, increased IL-4 and reduced oxygen free radicals in circulating monocytes. It thus appears that SHR respond to ROSU by antioxidant and anti-inflammatory processes. Our results are in accordance with other *in vitro* and *in vivo* studies which demonstrated the vascular antioxidant properties of statin therapy [61,62].

Because the cardiovascular protective effects of statins have also been observed in patients with normal cholesterol levels, it has been proposed that these compounds have a broad range of cholesterol-independent protective effects. Recent evidence has suggested that statins serve as regulators in the immune system [63]. In our study, ROSU lessened the expression of Th1 response IL-1α and IFN-γ and promoted the Th2 cytokine IL-4. These results are in accordance with the studies reported by Youssef et al. [64]. It follows that statins have many favourable effects on T-lymphocytes, which include reducing their cytotoxicity [65]. Atorvastatin is known to attenuate the Th1 immune response [66] and to diminish T-cell proliferation [67]. It is also conceivable that the improvement in endothelial function together with antioxidant and anti-inflammatory actions may have contributed to beneficial effect of ROSU.

In conclusion, the present study demonstrated that ROS and inflammation markers were inter-related in the development of hypertension in the SHR model and treatment with ROSU has beneficial effects in SHR by reducing oxidative stress status in plasma, circulating cells and aorta tissue. A combination of statins with drugs that primarily act through the restoration of vascular endothelial function associated with the limitation of oxidative stress, such as ACE inhibitors or angiotensin II receptor antagonists may provide additional benefits in patients at high cardiovascular risk. Further research into this possibility is necessary.

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