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Does Oxidative Stress Play a Critical Role in Cardiovascular Complications of Kawasaki Disease?

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Abstract

The aim of the present work was to evaluate the contribution of the different reactive oxidizing species to systemic oxidative stress in the whole blood of patients with Kawasaki disease (KD). This is a rare generalized systemic vasculitis typical of the early childhood characterized by inflammation and endothelial dysfunction with a high risk for cardiovascular fatal events. We found that, compared to age-matched healthy donors, blood from KD patients showed increased production of oxygen- and nitrogen-derived species as detected by electron paramagnetic resonance (EPR) spin probing with the cyclic hydroxylamine 1-hydroxy-3-carboxy-pyrrolidine. The $^{\bullet}$NO pathway involvement was also confirmed by the decreased concentrations of the endogenous $^{\bullet}$NO synthase inhibitor asymmetric dimethyl-arginine and the increased amounts of 3-nitrotyrosine in plasma. Further, increased plasma yields of the proinflammatory enzyme myeloperoxidase were also observed. The appearance of circulating red blood cell alterations typically associated with oxidative imbalance and premature aging (*e.g.*, decrease of total thiol content, glycophorin A, and CD47 expression, as well as increase of phosphatidylserine externalization) has also been detected. Collectively, our observations lead to hypothesize that the simultaneous oxidative and nitrative stress occurrence in the blood of KD patients may play a pathogenetic role in the cardiovascular complications often associated with this rare disease. *Antioxid. Redox Signal.* 17, 1441–1446.

Introduction

KAWASAKI DISEASE (KD) is a generalized systemic vasculitis typical of the early childhood (about 80% of cases occurring between 6 months and 5 years), which is characterized by inflammation of small- and medium-sized blood vessels, leading to a high propensity to damage of coronary arteries (1). The disease incidence is high in eastern countries (about 90 per 100,000 in Japan) and low in western countries (about 3–6 per 100,000), where it is considered as a rare disease.

The etiology of KD is still unknown, although both clinical and epidemiological findings strongly suggest that some infectious agent or bacterial superantigenic toxin can play a pathogenetic role (1). The major risk in KD progression is endothelial injury and coronary artery weakening, favoring the formation of aneurysms in 1:5 nontreated children with KD as well as myocardial infarction, ischemic heart, and sudden death (1). One of the potential cofactors playing a critical pathogenetic role in KD progression has been hypothesized to be represented

Innovation

Kawasaki disease is a rare generalized systemic vasculitis typical of the early childhood characterized by small- and medium-sized blood vessel inflammation also leading to coronary artery weakening, aneurysm formation, and myocardial infarction. This work adds new insights as concerns the implication of systemic oxidative stress in Kawasaki disease, that is, the simultaneous increase of $O_2^{\bullet}$- and $^{\bullet}$NO-derived species in *ex vivo* patient blood samples. These alterations probably contribute to disturbances of erythrocyte homeostasis resulting in anemia and formation of blood clots. These results reflect on a reappraisal of the redox status of plasma and circulating cells as bioindicators and targets in this often fatal childhood disease.

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by an inflammation-associated systemic pro-oxidant status (3). A modulation of some key biomarkers related to oxidative stress has in fact recently been demonstrated (9).

In this work, the occurrence of oxidative stress in plasma and red blood cells (RBCs) from patients with KD has been investigated with a particular regard to the oxidative and nitrative stress. Our data suggest that the presence of systemic oxidative imbalance could actually be pathogenetic in KD patients, also impairing RBC homeostasis, a common finding in patients with KD.

Oxidative Stress Is Increased in Whole Blood of KD Patients

The spin probe 1-hydroxy-3-carboxy-pyrrolidine (CPH) has been reported to be quickly oxidized by several oxidants ($O_2^{\bullet}$, $^{\bullet}NO_2$, peroxynitrite, transition metal-catalyzed reactions) to the corresponding stable 3-line-composed spectrum corresponding to the nitroxyl 3-carboxy-proxyl radical ($CP^{\bullet}$). We measured the rate of $CP^{\bullet}$ formation (vCP$^{\bullet}$) by adding CPH to air-equilibrated ($pO_2 = 205 \pm 15$ mmHg) whole blood of healthy donors (HD) and age-matched HD and KD patients. Figure 1A shows that the value of vCP$^{\bullet}$ measured in whole blood of KD patients was included in the reference control range measured in HD and age-matched HD (0.45–0.64 μM/min, mean value 0.53 ± 0.07 μM/min, $n = 45$) for 2 subjects (vCP$^{\bullet}$ of 0.52 ± 0.04 and 0.56 ± 0.01 μM/min, respectively), whereas it was significantly increased in blood of the other 6 subjects (vCP$^{\bullet}$ ranging from 0.71 to 1.46 μM/min), suggesting the occurance of a pro-oxidant activity in blood of some KD patients. The reactive oxidizing species (ROS), metal catalysis, and hemoprotein involvement in CPH oxidation in those KD patients showing CP$^{\bullet}$ formation in the upper range of Figure 1A ($n = 6$) was investigated by adding CPH to whole blood in the presence of superoxide dismutase (Sod), the $^{\bullet}$NO synthase inhibitor N-monomethyl-L-arginine (NMA), the metal chelator diethylenetriaminepentaacetic acid (DTPA), and the heme-binding compound KCN. DTPA, Sod, and NMA significantly decreased vCP$^{\bullet}$ in whole blood of KD patients by about 41%, 55%, and 24%, respectively, without affecting CP$^{\bullet}$ formation in whole blood of HD. KCN inhibited CP$^{\bullet}$ formation in whole blood of both HD and KD patients by about 26% and 74%, respectively. These results suggest that blood of KD patients is activated to produce simultaneously both $O_2^{\bullet}$- and $^{\bullet}$NO-derived species, which in turn mediates CP$^{\bullet}$ formation through a metal-catalyzed mechanism, with hemoproteins having a potential role.

The Concentrations of Oxidative Stress-Related Biomarkers Are Altered in Plasma of KD Patients

Since Sod- and NMA-dependent inhibition of CP$^{\bullet}$ formation in blood of KD patients could be an indication of peroxynitrite formation, we measured the involvement of this oxidant by measuring the formation of 3-nitrotyrosine (3-NitroTyr) in plasma of KD patients. Figure 1B shows that the concentration of 3-NitroTyr in plasma of seven KD patients was increased (range 0.109–0.204 μM) with respect to HD and age-matched HD (range 0.023–0.089 μM), suggesting that oxidizing species able to induce both tyrosyl radical and $^{\bullet}$NO-derived nitrating species were actually formed. The involvement of $^{\bullet}$NO-derived species was further investigated by measuring the plasma concentration of asymmetric dimethyl-arginine (ADMA), the endogenous inhibitor of $^{\bullet}$NO synthase. In plasma of HD and in age-matched HD, the ADMA concentration was within the range 0.46–0.69 μM, while it significantly decreased in six KD subjects (range 0.14–0.37 μM), suggesting an increased $^{\bullet}$NO production in the blood of KD patients (Fig. 1C). Finally, the KCN-mediated inhibition of CPH oxidation measured in whole blood of KD patients suggested the hemoprotein involvement. Beside Sod-inhibitable $O_2^{\bullet}$ – generating NADPH oxidase, in inflammed blood vessels of KD, activated leukocytes also release the pro-oxidant enzyme myeloperoxidase (MPO). Figure 1D shows that, with respect to HD and age-matched donors taken as reference control values (range 16.2–43.2 ng/ml), MPO concentration was (i) significantly increased in six patients (range 48.9–93.1 ng/ml), (ii) slightly increased in one patient (45.4 ± 1.3 ng/ml), and (iii) unchanged in one patient (41.2 ± 0.05 ng/ml). We underline that the modification of MPO, ADMA, and 3-Nitrotyr concentrations occurred simultaneously in the same six KD patients, suggesting that these compounds can be considered as real-time indicators of oxidative pathway activation in this disease.

Appearance of Aging and Death Biomarkers in RBCs of KD Patients

RBCs are considered the most important scavengers of ROS *in vivo* with a particular regard for $^{\bullet}$NO-derived species, that is, about ~50% of peroxynitrite. We previously reported that, in the reaction with RBCs, peroxynitrite can induce several morphological and functional modifications with the appearance of senescence (such as oxidation of thiols and downregulation of glycophorins) or apoptotic (clustering of band 3, externalization of phosphatidylserine [PS]) biomarkers. On the basis of these data and of the results reported in the previous paragraphs, intracellular redox balance (ROS production and total thiol content) and the expression of four different indicators of RBC alteration (glycophorin A [GA], CD47, band 3, and PS externalization) have been analyzed. GA is a glycoprotein widely expressed at the RBC surface that is downregulated during senescence. CD47 is an integrin-associated protein that is known as the thrombospondin receptor that acts as a marker of self. Band 3 is an ion exchanger, involved in RBC adhesion to endothelium. PS is a phospholipid normally localized to the inner leaflet of the plasma membrane, which is externalized to the outer leaflet during cell remodeling, leading to RBC aging and cell death. As reported in Table 1, a significant increase of ROS levels ($p < 0.01$) and a significant ($p < 0.05$) reduction of the intracellular content of total thiols were detected in RBCs from KD patients in comparison with those from HD. As concerns PS externalization, associated with the so-called eryptosis (7), a significantly ($p < 0.05$) increased percentage of RBCs showing translocation of this phospholipid at the outer leaflet of the plasma membrane was detected in cells from KD patients with respect to those from HD (Table 1). Moreover, analyzing GA, CD47, and band 3 by flow and static cytometry, a significant ($p < 0.01$) decreased expression and a redistribution of these proteins have been detected in RBCs from KD patients (Fig. 1E–G). Importantly, the appearance of these aging and death biomarkers on RBCs from KD patients was related with clinical evaluations. In fact, we found that during the first 5 days of hospitalization, the number of RBCs and the values of hemoglobin (Hb), mean corpuscolar volume, and hematocrit were significantly decreased in KD patients (Table 2).

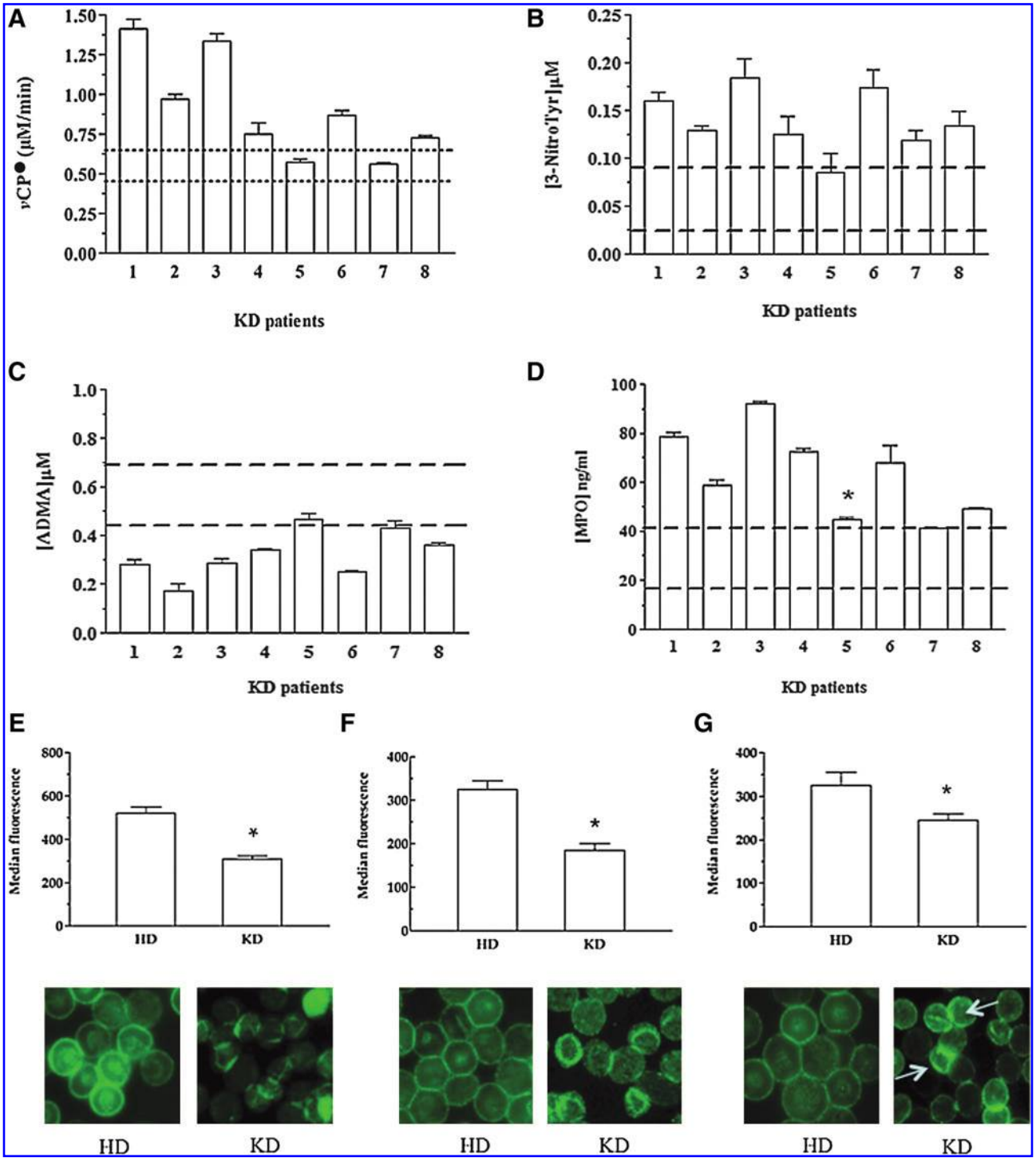

FIG. 1. Evidence for oxidative stress occurring in blood of KD patients. (A) The rate of CP• formation clearly shows that oxidative stress is increased in whole blood of KD patients. CP• formation was measured in triplicate as a function of time after the addition of CPH (1 m*M*) to air-equilibrated whole blood from HD ($n = 40$) and KD patients ($n = 8$). The dashed lines report the range of CP• measured in whole blood of HD. *$p \leq 0.05$, and all the other results were significant with $p \leq 0.001$ *versus* *v*CP• mean value ± SD of HD. Concentrations of 3-Nitrotyr **(B)**, ADMA **(C)**, and MPO **(D)** in blood plasma of KD patients. The concentrations of these biomarkers have been measured in triplicate in plasma of KD patients ($n = 8$) and compared to the concentrations of the relative biomarker measured in triplicate in HD ($n = 40$; dashed lines). *$p \leq 0.05$; all the other results were significant with $p \leq 0.001$ *versus* the biomarker mean values ± SD measured in HD. The biomarkers have been measured in blood plasma according to the manufacturer's instructions. Cytometric analyses clearly demonstrated a significant ($p < 0.01$) decreased expression of GA **(E)**, CD47 **(F)**, and band 3 **(G)** in RBCs from KD patients with respect to HD when analyzed by flow (histograms) and static (immunofluorescence micrographs) cytometry. Numbers represent the median values of fluorescence intensity and were obtained by analyzing RBCs from eight KD patients and five age-matched HD. The immunofluorescence micrographs are representative of one KD patient and one HD and show different arrangement and positivity of these molecules. In particular, a clustering of band 3 has been observed (*arrows*). ADMA, asymmetric dimethyl-arginine; CPH, 1-hydroxy-3-carboxy-pyrrolidine; CP•, 3-carboxy-proxyl radical; GA, glycophorin A; KD, Kawasaki disease; HD, healthy donors; MPO, myeloperoxidase; 3-NitroTyr, 3-nitrotyrosine; RBCs, red blood cells. (To see this illustration in color the reader is referred to the web version of this article at www.liebertpub.com/ars.)

Table 1. Redox Balance and Aging Markers in Red Blood Cells from Kawasaki Disease Patients

	Healthy donors	*KD patients*
ROS (MFI)	72 ± 2	102 ± 4[a]
Total thiols (MFI)	51 ± 3	40 ± 2[b]
PS externalization (%)	0.07 ± 2	0.19 ± 2[b]

[a] $p < 0.01$ *versus* healthy donors.
[b] $p < 0.05$ *versus* healthy donors.
KD, Kawasaki disease; ROS, reactive oxidizing species; MFI, median fluorescence intensity; PS, phosphatidylserine.

Conclusions and Open Questions

KD vasculopathy is characterized by accelerated carotid atherosclerosis and arterial stiffness, abnormal tissue doppler images, as well as increased cardiovascular risk (1, 3). In this work, we correlated blood ADMA levels with those of other biomarkers of oxidative stress, such as *v*CP$^{\bullet}$, and concentrations of 3-NitroTyr and MPO. We hypothesize that the occurrence of oxidative stress in blood of KD patients could likely be mediated by both ROS- and hemoprotein-derived oxidants. This hypothesis was supported by the finding that in blood of KD patients, (i) the rates of CPH spin-probe oxidation were increased and affected by ROS scavenger/inhibitor and metal chelators; (ii) the amounts of $^{\bullet}$NO-correlated biomarkers were modified; and (iii) the amounts of MPO were increased. These data suggest that oxidative and nitrative stress can simultaneously occur in blood of KD patients with $^{\bullet}$NO-derived species exerting a major role. It is important to underline that intravenous immunoglobulin treatment of KD patients decreased the appearance of oxidative stress biomarkers, in particular those related to $^{\bullet}$NO-pathway (inducible nitric oxide synthase expression and $^{\bullet}$NO-derived metabolites concentration), decreasing $^{\bullet}$NO-mediated inflammatory responses and coronary artery dilation (4). We can thus hypothesize that the systemic nitrosative imbalance can represent an early pathogenetic biomarker of the disease, which can be counteracted by intravenous immunoglobulin treatment. Furthermore, the oxidation of CPH in blood of KD was strongly inhibited by KCN with respect to HD, suggesting a major prooxidant role of hemoproteins (NADPH oxidase and MPO) in the disease progression. MPO can be then considered as an indicator of the proinflammatory status potentially able to participate to oxidative reactions leading to the 3-NitroTyr formation, as previously reported for some acute and chronic vascular and pulmonary diseases (2). In addition, hemoprotein-derived oxidants should be considered as further actors in the damage of host tissue, inducing metal release as a consequence of the oxidant reaction with tissue hemoproteins. This mechanism, which could explain why DTPA strongly decreased CPH oxidation in blood of those KD patients with high plasma MPO concentration, may contribute to vascular endothelial dysfunction occurring in this disease.

The appearance of biomarkers of aging and death detected in RBCs is a further important point. We found significantly decreased expression and redistribution of GA, CD47, and band 3; increased PS externalization at the RBC membrane; as well as an increased pro-oxidant status (increase of ROS and decrease of reduced thiols) in the cytosol. RBCs are physiologic scavengers of ROS in circulating blood and are considered either biosensors, monitoring oxidative imbalance in inflammatory diseases, or targets of systemic oxidative imbalance (8). Alterations in RBC structure and function may independently and synergistically impair blood flow and induce vascular occlusion, whereas premature aging of RBCs, and their consequent removal from circulation, might be a risk factor for anemia. In fact, both vascular occlusion and anemia are conditions that can be found KD patients (1, 6). Hence, on the basis of the results reported here, a reappraisal of RBCs as pathogenetic determinants and disease biomarkers appears as mandatory. Fittingly, it has been shown that intravenous immunoglobulin treatment restored normal hematocrit values in KD patients (5). This work provides new lines of evidence supporting the hypothesis that systemic oxidative stress and autocrine properties of MPO, together with premature aging of RBCs (this work) and platelets (9), could play a critical role in the cardiovascular risk observed in patients with KD.

Notes

Selection of KD patients and blood sampling

The investigation conforms with the principles outlined in the Declaration of Helsinki. A total of eight pediatric patients with KD (Orpha 2331), aged between 6 and 24 months and five HD age matched, have been recruited from the Bambino Gesù Hospital of Rome (Italy) and enrolled in this study. Diagnosis of KD was based on the classic clinical criteria. Laboratory data showed an increase of the erythrocyte sedimentation rate (79.3 ± 0.3 mm), C-reactive protein (8.9 ± 0.7 mg/l), and fibrinogen (444.5 ± 0.2 mg/dl). A single case presented hyperechogenicity areas of coronary arteries. All patients have been studied before starting any therapy with intravenous immunoglobulin and aspirin. The platelet count was 428.32 ± 0.5 ml. Heparinized fresh human whole blood was obtained from KD patients following informed consent by parents. This study was approved by the Institutional Review Board of Bambino Gesù Hospital of Rome, Italy. Whole blood was transferred in

Table 2. Hematologic Parameters

	First day of hospitalization	*Third day of hospitalization*	*Fifth day of hospitalization*	*Normal range for 6–24-month-old children*	
RBC ($\times 10^6/\mu$l)	4.4 ± 0.9[a]	4.2 ± 0.5	3.9 ± 0.2[a]	4 ± 5	[a] $p < 0.05$
Hb (g/dl)	11.4 ± 0.9[a]	10 ± 0.8	9.3 ± 0.7[a]	12.5 ± 1.5	[a] $p < 0.05$
MCV (fL)	74 ± 5[a]	70 ± 8	70 ± 6[a]	81 ± 6	[a] $p < 0.05$
Hematocrit (%)	33 ± 5[a]	30 ± 4	29 ± 2[a]	33 ± 9	[a] $p < 0.05$

[a] $p < 0.05$ fifth day of hospitalization *versus* first day of hospitalization.
RBC, red blood cell; Hb, hemoglobin; MCV, mean corpuscular volume.

15-ml Falcon and equilibrated in air on rocking for 10 min at room temperature. No appreciable cell lysis was observed during whole-blood air-equilibration as revealed by the lack of appearance of the characteristic Hb spectrum in blood plasma.

Isolation of RBCs

Fresh human blood from HD and Kawasaki patients was drawn into heparinized tubes. For RBC isolation, whole blood was centrifuged for 10 min at 1500 *g*. The plasma and buffy coat were removed; RBCs were washed twice in isotonic PBS, pH 7.4, and resuspended in the same buffer to the initial hematocrit concentration. No appreciable cell lysis was observed during the RBC preparation procedure.

Analysis of RBC redox balance

To evaluate intracellular reactive oxygen intermediates, RBCs (5×10^5 cells) were incubated in the Hanks' balanced salt solution, pH 7.4, containing dihydrorhodamine 123 (DHR 123; Molecular Probes). Intracellular content of total thiols was explored by using 5-chloromethylfluoresceindiacetate (CMFDA; Molecular Probes). Samples were then analyzed with a fluorescence-activated cell-sorting (FACS) flow cytometer (Becton Dickinson). The median values of fluorescence intensity histograms were used to provide semiquantitative evaluation of reduced thiol content and ROS production.

Analytical cytology

RBCs were fixed with 3.7% formaldehyde in PBS (pH 7.4) for 10 min at room temperature and washed in the same buffer. They were then permeabilized with 0.5% Triton X-100 in PBS for 5 min at room temperature. After washing with PBS, samples were stained with monoclonal anti-GA (Sigma-Aldrich), monoclonal anti-band 3 (Sigma), and monoclonal anti-CD47 (Santa Cruz Biotechnology). After 30 min at 37°C, samples were washed and then incubated for 30 min at 37°C with fluorescein isothiocyanate (FITC)-labeled anti mouse (Sigma). Secondary antibody given alone was used as negative control. Samples were analyzed by an Olympus BX51 Microphot fluorescence microscope or by flow cytometry with a FACScan flow cytometer (Becton Dickinson) equipped with 488-nm argon laser. At least, 20,000 events have been acquired. The median values of fluorescence intensity histograms were used to provide a semiquantitative analysis. The values of the untreated RBCs were set to 100%, and the levels of the considered proteins in the different samples were reported relative to these.

Evaluation of RBC injury

RBC eryptosis was quantitavely evaluated by flow cytometry after the double-staining method using the FITC-conjugated Annexin V apoptosis detection kit and 0.05% Trypan blue for 10 min at room temperature and analyzed by FACScan in the FL3 channel.

Determination of plasma 3-NitroTyr, ADMA, and MPO concentrations

Plasma MPO and ADMA concentrations were quantified using a commercial Stressgen EIA kits (Enzo Life Sciences, Inc.) according to the manufacturer's instructions. Plasma 3-NitroTyr concentration was quantified by ELISA.

EPR measurements

EPR spectra were measured at 37°C on a Bruker ECS 106 spectrometer (Bruker) equipped with a variable temperature unit (ER4111VT). Samples were drawn up into a gas-permeable Teflon tube with 0.81-mm internal diameter and 0.05-mm wall thickness (Zeuss Industrial Products). The Teflon tube was folded four times, inserted into a quartz tube, and fixed to the EPR cavity (4108 TMH). The dead time of sample preparation and EPR analysis was exactly 1 min after the last addition. The low-field shoulder has been chosen to quantify the $CP^{\bullet}$ radical, because the middle component centered at g 2.0 overlaps with many other free radical signals found in biological systems. The rate of $CP^{\bullet}$ formation ($v CP^{\bullet}$) was linear in the first 30 min of acquisition in all samples monitored. Spectrometer conditions common to all spectra were modulation frequency, 100 kHz; microwave frequency, 9.4 GHz; microwave power, 20 mW; gain 1×10^4; modulation amplitude, 1 G; conversion time, 20.5 ms; time constant, 82 ms; sweep time, 21 s; and number of scans, 1.

Statistical analyses

Cytofluorimetric results were statistically analyzed by using the nonparametric Kolmogorov–Smirnov test using Cell Quest Software. At least 20,000 events were acquired. The median values of fluorescence intensity histograms were used to provide a semiquantitative analysis. Statistical analyses of collected data were performed by using Student's *t*-test.

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Abbreviations Used

ADMA = asymmetric dimethyl-arginine
CMFDA = 5-chloromethylfluoresceindiacetate
$CP^{\bullet}$ = 3-carboxy-proxyl radical
CPH = 1-hydroxy-3-carboxy-pyrrolidine
DHR 123 = dihydrorhodamine 123
DTPA = diethylenetriaminepentaacetic acid
FACS = fluorescence-activated cell sorting
FITC = fluorescein isothiocyanate
GA = glycophorin A
HDs = healthy donors
KD = Kawasaki disease
MPO = myeloperoxidase
3-NitroTyr = 3-nitrotyrosine
NMA = N-monomethyl-L-arginine
PS = phosphatidylserine
RBCs = red blood cells
ROS = reactive oxidizing species
Sod = superoxide dismutase