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Nitric oxide activity in platelets of dengue haemorrhagic fever patients: the apparent paradoxical role of ADMA and L-NMMA

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ABSTRACT

Dengue haemorrhagic fever (DHF) is a prevalent acute disease that occurs in patients infected by an arbovirus in tropical and subtropical regions. We have previously shown increased intraplatelet nitric oxide (NO) production in patients with dengue fever associated with reduced platelet aggregation. In this study, L-arginine transport as well as expression and activity of nitric oxide synthase (NOS) isoforms in the presence or absence of L-arginine analogues were examined in 23 DHF patients. L-arginine transport and NOS activity in platelets were increased in patients with DHF compared with controls. However, platelet endothelial NOS (eNOS) and inducible (iNOS) protein levels did not differ between healthy controls and DHF patients. Endogenous or exogenous analogues did not inhibit platelet NOS activity from DHF patients. In contrast, endogenous L-arginine analogues [N^G-monomethyl-L-arginine (L-NMMA) and asymmetric dimethylarginine (ADMA)] inhibited NOS activity in platelets from healthy subjects. These results show the first evidence that the intraplatelet L-arginine–NO pathway is activated in DHF patients. The lack of inhibition of NO formation in vitro by all L-arginine analogues tested in DHF platelets may suggest another mechanism by which NOS activity can be regulated.

1. Introduction

Dengue fever (DF) is a mosquito-borne human disease caused by any one of the four genetically related but distinct dengue virus serotypes (DENV-1, DENV-2, DENV-3 and DEN-4).[1] Infection by dengue virus may be asymptomatic or may lead to a broad spectrum of clinical manifestations, ranging from unspecific fever (an influenza-like symptom known as DF) to a severe, sometimes fatal, disease characterised by haemorrhage and shock known as dengue haemorrhagic fever (DHF).[1] Dengue infection has become a major public health issue in tropical and subtropical countries, with an estimated 50 million infections per year worldwide, including 250 000–500 000 cases of DHF with a case fatality rate up to 20% in the absence of treatment.[2] In Brazil, DHF occurs predominantly in adults, although mortality rates are higher in children. In 2008, there was a dengue outbreak

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in Brazil, specifically in Rio de Janeiro State. More than 250 000 cases of DF, including almost 2000 cases of DHF, with 252 deaths were recorded.[3]

The clinical diagnosis of DHF is based on four major clinical manifestations: (i) high fever lasting for 2–7 days; (ii) haemorrhagic tendency, such as a positive tourniquet test, petechiae or epistaxis; (iii) marked thrombocytopenia (platelet count $\leq 100 \times 10^9$/l); and (iv) plasma leakage evidenced by haemoconcentration (an increase in haematocrit $\geq$20% above average for age, sex and population), pleural effusion and ascites.[4]

The pathogenesis of DHF is still poorly understood. It has been suggested that DHF might be due to the occurrence of an abnormal immune response involving production of cytokines, activation of T-cells[5] and disturbance of the haemostatic system.[6] Cytokines generated by antibody-mediated dengue infection of monocytes or released by dengue-infected monocytes or macrophages increase endothelial cell permeability, which may lead to plasma leakage.[7]

The haemorrhagic complications are a consequence of the low platelet count-associated vasculopathy, coagulopathy and platelet dysfunction. The marked thrombocytopenia observed in DHF patients, with a platelet nadir as low as 5×10^9cells/l, appears to be caused by early bone marrow suppression combined with increased peripheral destruction of platelets during febrile and early convalescent phase of dengue disease.[8] Another hypothesis to explain the thrombocytopenia is an increase in platelet adhesion to endothelial cells.[9] In addition to a low platelet count, there is an alteration in platelet function. Platelets from DHF patients show an impaired aggregation response to adenosine diphosphate (ADP) during the febrile and early convalescent stages.[10]

Nitric oxide (NO) is a gaseous messenger with physiological and pathological implications. NO was first described as a potent vasodilator produced by endothelial cells and plays a physiological role in maintaining haemostasis, regulating vasodilatation, and aggregation, recruitment and adhesion of platelets to the vascular endothelium.[11] NO is generated via oxidation of the cationic amino acid L-arginine, catalysed by a family of nitric oxide synthase (NOS) enzymes.[11] L-arginine transport is essential to NO synthesis in different cell types as it limits NOS activity.[12] Platelets express both inducible and constitutive NOS isoforms.[13]

We have previously demonstrated that NOS activity and L-arginine transport in blood cells are inhibited by endogenous L-arginine analogues such as asymmetric dimethylarginine (ADMA) and N^G-monomethyl-L-arginine (L-NMMA), and this inhibition is involved in the pathophysiology of many diseases.[14–16] ADMA is considered a cardiovascular risk factor and is found at an elevated concentration in different diseases.[17] Paradoxically, L-arginine analogues have been tested as a pharmacological tool in septic shock, without success.[18]

Our group showed upregulation of the intraplatelet L-arginine–NO pathway in DF patients, associated with a decrease in platelet aggregation.[19] To our knowledge, there are few studies evaluating platelet function in DHF[10,20] and none investigating platelet production of NO.

In this study, L-arginine transport as well as activity and expression of inducible NOS (iNOS) and endothelial NOS (eNOS) in DHF platelets were investigated. In addition, the effects of endogenous and exogenous L-arginine analogues were analysed as possible pharmacological tools to inhibit platelet NO synthesis and, as a consequence, to reduce bleeding in DHF patients.

2. Materials and methods

2.1. Subjects

In total, 23 DHF patients and 25 age-matched healthy volunteers participated in this study (mean ± SD age, 33 ± 14 years and 34 ± 9 years, respectively; not significantly different). The patients were recruited from Hospital Universitário Pedro Ernesto, Hospital Municipal Lourenço Jorge and Hospital de Ipanema (Rio de Janeiro, Brazil) during January–March 2008, and were clinically diagnosed with DHF grade I and II according to WHO criteria and serologically confirmed by ELISA anti-dengue IgM and IgG.[4] Only samples collected from patients within 7 days of fever onset were included in the study. Written informed consent was obtained from each of the patients. The majority of patients with DHF had a secondary infection and the platelet count (mean ± SD) was $46 \pm 10 \times 10^9$/l. The major haemorrhagic manifestations observed were petechiae (77%), epistaxis (33%), metrorrhagia (44%) and bleeding in the venipuncture site (88%). Healthy volunteers were recruited among students and staff of the State University of Rio de Janeiro (platelet count $277 \pm 36 \times 10^9$/l). The number of platelets was normalised in each experiment. The DHF patients and healthy volunteers who participated in the study were not the same in every experiment because for ethical reasons it is not possible to obtain the amount of blood needed.

2.2. Isolation of platelets

Blood samples were drawn by venipuncture into citric acid–dextrose-containing tubes [composition (in mmol/l): citric acid 73.7; trisodium citrate 85.9; dextrose 111] and were centrifuged at $250 \times g$ for 15 min at room temperature. Platelets were isolated from platelet-rich plasma by centrifugation at $800 \times g$ for 15 min at room temperature and the platelet pellet was re-suspended in Krebs' buffer [composition (in mmol/l): NaCl 119; KCl 4.6; $CaCl_2$ 1.5; NaH_2PO_4 1.2; $MgCl_2$ 1.2; $NaHCO_3$ 15; glucose 11]. Blood cells and platelets were counted in the platelet suspension using a cell counter (Automated Hematology Analyzer XT-1800i; Sysmex Corporation, Kobe, Japan). Potential contamination from other cell types was excluded as the leukocyte count was minimal ($<0.01 \times 10^6$/ml).

2.3. Platelet L-arginine influx

For influx experiments, 0.5 ml of the platelet suspension (1×10^9 platelets/ml) was incubated with L-[^{3}H]-arginine (37 kBq/ml) at 37 °C. Total transport was fractionated into diffusion and system y^+L by cis-inhibition with unlabelled