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Fibrinogen nitrotyrosination after ischemic stroke impairs thrombolysis and promotes neuronal death

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ABSTRACT

Ischemic stroke is an acute vascular event that compromises neuronal viability, and identification of the pathophysiological mechanisms is critical for its correct management. Ischemia produces increased nitric oxide synthesis to recover blood flow but also induces a free radical burst. Nitric oxide and superoxide anion react to generate peroxynitrite that nitrates tyrosines. We found that fibrinogen nitrotyrosination was detected in plasma after the initiation of ischemic stroke in human patients. Electron microscopy and protein intrinsic fluorescence showed that *in vitro* nitrotyrosination of fibrinogen affected its structure. Thromboelastography showed that initially fibrinogen nitrotyrosination retarded clot formation but later made the clot more resistant to fibrinolysis. This result was independent of any effect on thrombin production. Immunofluorescence analysis of affected human brain areas also showed that both fibrinogen and nitrotyrosinated fibrinogen spread into the brain parenchyma after ischemic stroke. Therefore, we assayed the toxicity of fibrinogen and nitrotyrosinated fibrinogen in a human neuroblastoma cell line. For that purpose we measured the activity of caspase-3, a key enzyme in the apoptotic pathway, and cell survival. We found that nitrotyrosinated fibrinogen induced higher activation of caspase 3. Accordingly, cell survival assays showed a more neurotoxic effect of nitrotyrosinated fibrinogen at all concentrations tested. In summary, nitrotyrosinated fibrinogen would be of pathophysiological interest in ischemic stroke due to both its impact on hemostasis – it impairs thrombolysis, the main target in stroke treatments – and its neurotoxicity that would contribute to the death of the brain tissue surrounding the infarcted area.

1. Introduction

Stroke is a leading cause of death and disability worldwide [1,2]. The effectiveness of ischemic stroke treatment depends on its rapid and accurate diagnosis [3]. The currently available treatment target is clot lysis with recombinant tissue plasminogen activator (rtPA) but carries a certain risk of bleeding and less than 5% of stroke patients receive this treatment due to the very narrow therapeutic window for rtPA (4.5 h after stroke onset) [4].

Ischemic stroke is an acute vascular event that hinders blood supply to the brain and leads to an ischemic process that affects neurons, glial cells and vessels. The tissue surrounding the ischemic core lesion is

Abbreviations: Ab, antibody; BSA, bovine serum albumin; CP, cortical perfusion; FBS, fetal bovine serum; GSH, reduced glutathione; MCA, middle cerebral artery; MG, methylglyoxal; MTT, 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NTyr, 3-nitrotyrosine; $O_2^{\cdot-}$, superoxide anion; $ONOO^-$, peroxynitrite anion; o.n., overnight; RT, room temperature; rt-PA, recombinant tissue plasminogen activator; SH-SY5Y, human neuroblastoma cells; SIN-1, 3-morpholinosydnonimine hydrochloride; SNP, sodium nitroprusside

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termed "penumbra", a region where neurons are still viable owing to residual blood perfusion [5]. In this scenario, the liberation of nitric oxide (NO), a molecule with pleiotropic effects in the brain [6], is increased to favor vasodilatation and blood supply to the compromised brain region [7], while reperfusion after thrombolysis induces a burst of free radicals such as the superoxide anion ($O_2^{-\cdot}$) [8,9]. NO reacts with O_2^- producing the highly reactive peroxynitrite anion ($ONOO^-$) [10], which, among other harmful effects, irreversibly nitrates proteins [11,12]. This process, known as nitrotyrosination, is a post-translational modification that normally leads to a loss of protein function.

Fibrinogen is one of the most abundant plasma proteins. Its main physiological function is hemostasis as a result of its aggregation to the fibrin polymers that mediate clot formation. Increased levels of circulating fibrinogen have been identified as a stroke risk factor [13] as well as a bad prognosis factor after stroke [14–16]. In the present work we have evaluated the relevance of fibrinogen nitrotyrosination in ischemic stroke, with particular emphasis on hemostasis and cell toxicity.

2. Materials and methods

2.1. Biological material

Human brain sections obtained from autopsies of patients who had an ischemic stroke were provided by the Servei d'Anatomia Patològica (Hospital del Mar, Barcelona) corresponding to 4 patients who were 68 ± 34 years old (2 men and 2 women). Human plasma and anticoagulant free whole blood samples, provided by the Servei de Neurologia (Hospital del Mar), were obtained from patients after ischemic strokes and from controls. Blood extraction was carried out approximately 3 h after ischemic stroke. Controls were 53 ± 4 years old (14 men and 9 women); atherothrombotic stroke patients were 70 ± 2 years old (8 men and 9 women); cardioembolic stroke patients were 78 ± 2 years old (5 men and 9 women); undetermined stroke patients were 72 ± 4 years old (2 men and 7 women); and lacunar stroke patients were 67 ± 4 years old (7 men and 5 women). All procedures were approved by the Ethics Committee of the Institut Municipal d'Investigacions Mèdiques — Universitat Pompeu Fabra. Subjects gave informed consent.

Plasmas from rat were obtained from 3-month old Sprague–Dawley rats. The procedure was approved by the Ethics Committee of the Universitat de Barcelona.

Human neuroblastoma cell line (SH-SY5Y), supplied by ECACC, were cultured with Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA).

2.2. Clot formation

Anti-coagulant free whole blood samples were treated immediately after extraction with phosphate-buffered saline (PBS; controls), sodium nitroprusside (SNP; a NO donor, Sigma, St. Louis, USA) + H_2O_2 or H_2O_2 (Sigma, St. Louis, USA). They were allowed to clot for 3 h at 37 °C. Clots were removed and embedded in optimal cutting temperature compound (Sakura Finetek) medium and frozen to −80 °C. The clots were analyzed by immunodetection as described below to study the structure of the fibrin network and the presence of nitrotyrosination.

2.3. Immunodetection

Formalin-fixed paraffin-embedded brain sections were cut at 3 μm, deparaffinated at 70 °C for 1 h and washed with decreasing concentrations of ethanol. Antigen retrieval was performed with proteinase K at 40 μg/mL in a 1:1 glycerol and TE buffer solution. Frozen clots were cut at 5 μm in a cryostat. Immunostaining was performed with 1:200 mouse monoclonal anti-3-nitro-tyrosine (NTyr; Cayman Chemical, Michigan, USA) antibody (Ab) or 1:200 rabbit polyclonal anti-human fibrinogen (Dako, California, USA) Ab for 2 h at room temperature (RT) followed by 1:1000 Alexa555-bound anti-mouse or 1:1000 Alexa488-bound anti-rabbit as secondary Abs (Dako, California, USA) overnight (o.n.) at 4 °C. Sections were stained with TO-PRO to identify the nuclei and mounted with Mowiol. Images were taken with a Leica TCS SP confocal microscope and analyzed with Leica confocal software (Leica).

SH-SY5Y cells (4×10^4 cells/well) were seeded on 1.5% gelatin-coated 12 mm coverslips. Cells were treated for 45 min with PBS (controls), 5 μg/μL fibrinogen (Sigma, St. Louis, USA) or 5 μg/μL nitrofibrinogen prepared by pre-incubating fibrinogen with 100 μM 3-morpholinosydnonimine hydrochloride (SIN-1; Sigma, St. Louis, USA), an $ONOO^-$ donor. They were fixed after 45 min (fibrinogen challenge) and incubated for 2 h at RT with 1:500 rabbit anti-cleaved caspase-3 (Asp175) Ab (Cell Signaling, Beverly, USA) followed by 1:500 Alexa Fluor 488 goat anti-rabbit polyclonal Ab for 1 h at RT. Cells were stained with TO-PRO to identify the nuclei and mounted with Mowiol. Images were taken with a Leica TCS SP confocal microscope and analyzed with Leica confocal software (Leica).

2.4. Focal cerebral ischemia in rats

Focal cerebral ischemia was produced by transient intraluminal occlusion of the middle cerebral artery (MCA) in rats, as previously reported [17]. Plasma (500 μL) was extracted from the rats after 3, 6, 12, and 24 h of MCA occlusion. The procedure was approved by the Ethics Committee of the Universitat de Barcelona (CEEA-273/09).

2.5. Fibrinogen immunoprecipitation

Human and rat plasma samples (350 μL) were incubated with 5 μg of anti-human fibrinogen polyclonal Ab or to 5 μg of anti-rat fibrinogen polyclonal Ab (Accu-Specs, Westbury, NY, USA). Samples were shaken overnight at 4 °C. Following addition of 20 μg of protein G-sepharose (Sigma, St. Louis, USA), samples were shaken for 2 h at 25 °C, centrifuged at 10,000 rpm for 10 min and washed 3 times with PBS.

2.6. Quantification of nitrotyrosination

Immunoprecipitated fibrinogen was used at a final volume of 100 μL. NTyr was determined spectrophotometrically by the absorbance measurement at 415 nm (pH > 9) [18]. A calibration curve using serial dilutions of free NTyr (Sigma, St. Louis, USA) was used to quantify nitrotyrosination expressed as NTyr μg/μL or mol of NTyr/mol of Fib.

2.7. Fibrinogen conformational state analysis by intrinsic fluorescence measurement

Intrinsic protein fluorescence emission is mainly due to tryptophan residues, which have a wavelength of maximum absorption of 280 nm and an emission peak ranging from 300 to 350 nm. Therefore the shift in protein fluorescence can be used to study the changes in protein conformational states [19]. Freeze-dried human fibrinogen was directly solubilized at 1 mg/mL in TBS (50 mM Tris base, 150 mM sodium chloride; pH 7.4). Fibrinogen was then incubated in the presence of 12 mM $CaCl_2$, with or without 100 μM SIN-1 at 37 °C for 24 h in the dark. Intrinsic fluorescence measurements of these mixtures were determined in a Shimadzu spectrofluorophotometer (RF-5301). Samples were excited at 280 nm and fluorescence emission reading was recorded between 300 and 400 nm.