Title page

Title: Reported pre-stroke physical activity is associated with VEGF expression and good outcomes after stroke

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Short title: Pre-stroke physical activity and VEGF expression

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Abstract

Introduction: Physical activity (PhA) prior to stroke has been associated with good outcomes after the ischemic insult, but there is scarce data on the involved molecular mechanisms. **Methods:** We studied consecutive acute ischemic stroke patients admitted to a single tertiary stroke center. Pre-stroke PhA was evaluated with the International Physical Activity Questionnaire (METs-minute/week). We studied several circulating angiogenic and neurogenic factors at different time-points: Vascular Endothelial Growth Factor (VEGF), Granulocyte Colony-Stimulating Factor (G-CSF) and Brain-Derived Neurotrophic Factor (BDNF) at admission, day 7, and at 3 months. We considered good functional outcome at 3 months (mRS ≤ 2) as primary endpoint, and final infarct volume as secondary outcome. **Results:** We studied 83 patients with at least two time-point serum determinations (mean age 69.6 years, median NIHSS 17 at admission). Patients more physically active before stroke had a significantly higher increment of serum Vascular Endothelial Growth Factor (VEGF) at 7th day when compared to less active patients. This increment was an independent predictor of good functional outcome at 3 months and was associated with smaller infarct volume in multivariate analyses adjusted for relevant covariates. We did not find independent associations of G-CSF or BDNF levels neither with level of pre-stroke PhA nor with stroke outcomes. **Conclusions:** Although there are probably more molecular mechanisms by which physical activity exerts its beneficial effects in stroke outcomes, our observation regarding the potential role of VEGF is plausible and in line with previous experimental studies. Further research in this field is needed.

Key words: functional recovery, physical activity, stroke, vascular growth factors

Introduction

Brain ischemic tolerance refers to the concept that some stimuli prior to ischemia may lead to a greater tolerance to the subsequent injury. Physical activity has been proposed as a good preconditioning stimulus (1) and has been also related to brain repair after ischemia (2). Animal models of cerebral ischemia have shown that pre-stroke physical activity (PhA) is associated with reduced functional deficits, smaller infarct volume and better functional recovery. Several molecular mechanisms in animal models have been related to these neuroprotective effects such as: increase in cerebral blood flow mediated by a higher expression of endothelial Nitric Oxide Synthase (NOS) (3,4); improvement on blood-brain-barrier (BBB) function; upregulation of angiogenesis via expression of vascular and neural growth factors (1) and endothelial progenitor cells mobilization (5), among others.

We have previously reported that higher levels of self-reported pre-stroke PhA were independently associated with a better functional outcome at three months and with a smaller final infarct volume in a cohort of acute stroke patients with cerebral anterior circulation large artery occlusion (6). However, there is not clear clinical evidence about the molecular mechanisms involved in the beneficial effect of PhA so far. Based on the previous preclinical studies (3-5) and on our previous clinical findings (6), our aim was to evaluate possible molecular pathways associated with pre-stroke PhA and with stroke outcomes, by studying some angiogenic and neurogenic growth factors at different timepoints after stroke onset.

Methods

The present paper is a secondary study of AFRICA (Prestroke Physical Activity and Functional Recovery in patients with IsChemic stroke and Arterial Occlusion) study (6). AFRICA is an observational and longitudinal study performed in consecutive acute stroke patients admitted at our tertiary stroke center (Germans Trias i Pujol University Hospital, Universitat Autònoma de Barcelona, Spain) between June 2008 and January 2011. Study protocol has been detailed elsewhere (6). Briefly, specific inclusion criteria to enter this study were: patients with acute ischemic stroke of anterior circulation within 12 hours from symptoms onset; a confirmed occlusion of middle cerebral artery (MCA) or terminal intracranial carotid artery (TICA) upon admission; and functional independence defined as modified Rankin scale (mRS) ≤ 1 prior to stroke. In the acute phase, patients were treated or not with reperfusion therapies (intravenous thrombolysis and/or endovascular treatment) following internal protocols based on national and international guidelines. Demographic and clinical variables were registered in specific case reporting forms. Stroke severity was assessed with the National Institute of Health Stroke Scale (NIHSS). Distal occlusion was considered if occlusion of M2-MCA segment. This research was carried out in accordance with the Declaration of Helsinki of the World Medical Association and approved by the Ethics Committee of our institution. Written informed consent was obtained from each patient or their relatives after full explanation of the procedures.

Serums were obtained from blood samples drawn at admission, 7th day and 3rd month after stroke onset in glass chemistry tubes, which were centrifuged at 3,000 g during 10 minutes, and immediately frozen and stored at -80°C. Serum levels of VEGF, G-CSF and BDNF (Boster Biological Technology, Encyclopedia Circle Fremont, CA, USA) were measured using commercial ELISA kits following manufacturer instructions. Serum determinations of molecules were performed in an external laboratory part of our research network

(Clinical Neurosciences Research Laboratory, University of Santiago de Compostela), by a biologist blinded to clinical data. Coefficients of variation intra-assay were 4.4%, 5.2% and 4.8% and inter-assay were 6.9%, 8.3% and 7.9%, respectively.

Patients or direct relatives underwent a complete interview to assess regular PhA prior to stroke.

Level of pre-stroke PhA was evaluated using the internationally validated IPAQ (International Physical Activity Questionnaire) (7). Interviews were made in the first 48 hours after admission by an investigator blinded to clinical data. IPAQ questionnaire records the PhA for the week before the interview is conducted. In the present study, we asked about the last week prior to admission to the hospital. With IPAQ, computation of the total score requires summation of the duration (in minutes) and frequency (days) for all the types of activities in all domains (leisure, domestic, work-related and transport-related) and is represented in metabolic equivalent (MET)-minutes per week. For main multivariate analyses we considered IPAQ score as a continuous variable. We also dichotomized level of pre-stroke PhA as low-moderate versus high following predefined thresholds of the scale: a high level of pre-stroke physical activity is considered with any combination of walking, moderate or vigorous-intensity activities achieving a minimum of at least 3000 MET-minutes/week. Primary outcome variable was functional outcome, with good outcome defined as a modified Rankin Scale (mRS) ≤ 2 at 3 months after stroke. Secondary outcome was final infarct volume. Final infarct volume was measured as $A \times B \times C / 2$ (maximal diameters in mm) of the cerebral hypodensity on CT scan performed 30 days after stroke by an investigator blinded to clinical data.

Statistical analysis

First, we evaluated the serum levels of the molecules according to both level of pre-stroke PhA (high versus moderate-low) and functional outcome (good versus poor). Differences in levels of growth factors between groups at each time-point were compared with the Student's test. Biomarkers significantly associated with pre-stroke PhA and with stroke outcomes and their changes between time-points (increment or decrement) were selected for further analyses. Thereafter, we constructed logistic and linear regression models to evaluate the independent association of relevant serum biomarkers with good functional outcome at three months and with final infarct volume respectively. We used logarithmic transformation of IPAQ score and final infarct volume for linear regression analyses. Multivariate models were adjusted for pre-stroke PhA and other relevant covariates significantly associated with outcomes in univariate analyses, using forward and backward steps. Results were expressed as adjusted odds ratios (OR) and linear regression coefficients (B) with the corresponding 95% confidence intervals (95% CI). We evaluated relevance of including circulating markers in the models as change of area under the curve (AUC) in logistic regression analysis and change of R^2 in linear regression analysis. Statistical analysis was conducted using SPSS 18.0 (SPSS Inc. Chicago, IL USA) for Windows XP.

Results

From the original AFRICA cohort of 159 patients, there were available serum samples of 126 patients at admission and of 83 patients at 7th day after stroke. Main baseline and outcome variables were similar between the original AFRICA cohort and the subsets of patients with available samples (Supplemental Table 1). In a sensitivity analysis, we evaluated circulation levels of VEGF, G-CSF and BDNF at admission in the whole subset

of 126 patients, and there were no significant associations neither with prior PhA nor with stroke outcomes. Therefore, we included the 83 patients with at least two time-point serum determinations (admission and 7th day) for further analyses.

Table 1 shows baseline characteristics and levels of neurogenic and angiogenic factors at different time-points in the global sample and in both groups of pre-stroke PhA level. At admission, VEGF, G-CSF and BDNF levels were similar among patients with low-moderate level compared to those with high level of pre-stroke PhA, but the latter had significantly higher levels of VEGF at 7th day when compared to those with a low-moderate level of PhA (99.7 ± 41.4 vs 73.0 ± 39.9 pg/mL, $p < 0.01$; mean difference 95% CI [-45.5, -7.8]). Therefore, increment of VEGF from baseline to 7th day was significantly higher in patients more physically active previous to the ischemic insult (mean increment 30.1 vs 14.4 pg/ml, $p < 0.05$). Increment of VEGF in the first 7 days was negatively correlated with stroke severity (NIHSS) at admission (-0.31 , $p = 0.004$) and at 7th day (-0.65 , $p < 0.001$). Interestingly, patients that reached a good functional outcome at three months also had significantly higher levels of VEGF at 7th day when compared to patients with poor outcome (100.4 ± 39.8 vs 65.1 ± 37.0 pg/mL, $p < 0.01$; mean difference 95% CI [-52.1, -18.5]) (Table 2). Changes in VEGF levels in the first 7 days according to level of pre-stroke PhA and to functional outcome at 3 months in individual patients are represented in **Figure 1**. Three months after stroke, levels of VEGF were still significantly higher in patients that reached a good outcome (Table 2). However, at that time there were available samples only in 62 patients, and the 21 patients without available samples had a poorer prognosis (mRS > 2 in 81% vs 43.5% of patients).

G-CSF levels were significantly higher at 7th day in those with a good outcome but were not associated with level of pre-stroke PhA. BDNF levels were similar along the three timepoints, regardless of pre-stroke PhA level or functional outcome after stroke.

In logistic regression analysis, both pre-stroke PhA and increment of VEGF at 7th day (VEGF at 7th day-VEGF at admission) were independently associated with good functional outcome at three months (Table 3). AUC was higher when VEGF was included in the model (0.914 if only pre-stroke PhA was included and 0.946 when both variables were included). In linear regression analysis, both high level of pre-stroke PhA and increment of VEGF at 7th day showed an inverse association with final infarct volume (Table 4). In this case, including VEGF in the model slightly changed R² from 0.48 to 0.50%.

Discussion

In the present study we have observed that a high level of self-reported physical activity prior to stroke was associated with greater VEGF expression in the first days after ischemic stroke. Likewise, this increment in VEGF levels was independently associated with a reduction in final infarct volume and with improvement of functional outcome at three months.

The role of vascular endothelial growth factors in neuroprotection, neurogenesis, angiogenesis and postischemic brain and vessel repair has been extensively studied (8). Brain VEGF expression is induced in the ischemic penumbra during stroke and increases over days to weeks following MCA occlusion in rats (9), and we have observed similar results in circulating levels in our patients. Analogous to our findings, VEGF expression

has been associated with smaller cerebral infarcts after MCA occlusion in transgenic rats (10), and VEGF-B knockout mice developed larger infarct volumes and more severe neurological deficits compared to wild-type mice (11). Moreover, VEGF is thought to be an important mediator of NO-induced angiogenesis in the postischemic brain (12). Also, VEGF mediates the mobilization of circulating endothelial progenitor cells (EPCs) during the first hours and days after stroke (13), which appear to have an important role in repairing blood vessels after ischemic injury. Interestingly, VEGF expression is also activated in remote sites to the ischemic tissue, especially in areas related to the motor function affected by the infarct (14). Unfortunately, studies using VEGF delivery as a treatment for enhancing post-stroke angiogenesis revealed not only positive effects but also negative effects such as BBB leakage and brain edema, especially when administered intravenously (15). The enhancement of VEGF expression by physical activity has also been observed in experimental studies, similar to our findings (16). In this regard, the VEGF-stimulation via physical activity may represent a potential and safe alternative against the exogenous VEGF treatment.

Our study has limitations that should be mentioned. The main limitation is that biomarkers were not measured in the entire AFRICA study cohort; however, main baseline and outcome variables in patients with available samples were similar to those in the original study. Also, the fact that evaluation of pre-stroke PhA is based on a subjective questionnaire may introduce bias about the real PhA level performed by patients; however, IPAQ is an internationally validated instrument and the use of a questionnaire is inherent to the observational nature of our study. Some important variables related with stroke outcomes (baseline NIHSS, glucose levels and reperfusion treatments) were significantly

unbalanced among groups of level of pre-stroke PhA, a fact that we were not able to control due to consecutive enrollment of patients. We tried to overcome this limitation including these variables in the multivariate models. Our study has some advantages, such as the prospective design with consecutive enrollment of patients, the homogenous sample including anterior circulation strokes with large artery occlusion and the use of standardized questionnaires to assess PhA.

Although there are probably more molecular mechanisms by which physical activity exerts its beneficial effects in stroke outcomes, our observation regarding the potential role of VEGF is plausible and in line with previous experimental studies. Further research in this field is needed.

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Conflict of interest

Authors report no conflicts of interest

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Title and legends to figures

Figure 1:

Title: Changes in VEGF in the first 7 days according to level of pre-stroke PhA and to functional outcome for each patient.

Legend: In horizontal axis, dots represent levels of VEGF at admission in individual patients. Patients with a moderate-low level of pre-stroke PhA are represented on the left part of the graph and those with a high level of PhA on the right. From each point, an arrow shows the increase or decrease of VEGF levels at 7th day. Green arrows represent good outcome and red arrows poor outcome at three months.

Table 1: Baseline clinical characteristics and angiogenic/neurogenic factors in the global sample and in groups of pre-stroke PhA

Table 1	Global N=83	Low-moderate level prestroke PhA N=56	High level prestroke PhA N=27	P
Age, years	69.6±10.9	71.0 ±10.6	66.9±11.4	0.115
Males, n (%)	48 (57.8)	33 (58.9)	15 (55.6)	0.771
Smoking habit, n (%)	14 (16.9)	8 (14.3)	6 (22.2)	0.366
Hypertension, n (%)	56 (67.5)	41 (73.2)	15 (55.6)	0.108
Diabetes, n (%)	17 (20.5)	12 (21.4)	5 (18.5)	0.758
Glucose levels at admission mg/dl	126.1±37.9	132.4±37.3	113.1±36.3	0.028
NIHSS at admission	17 [12-21]	18 [14-22]	13 [7-19]	0.002
Proximal artery occlusion, n (%)	54 (65.1)	40 (71.4)	14 (51.9)	0.080
Reperfusion therapies administered, n(%)	64 (77.1)	39 (69.6)	25 (92.6)	0.020
- Intravenous rTPA	37 (57.8)	20 (35.7)	17 (63)	
- I.V rTPA+endovascular	14 (21.9)	10 (17.7)	4 (14.8)	
- Endovascular	13 (20.3)	9 (16.1)	4 (14.8)	
Angiogenic/neurogenic factors				
VEGF at admission (pg/mL)	62.2±39.3	58.6±37.6	69.6±42.4	0.236
VEGF at 7th day (pg/mL)	81.7±41.8	73.0±39.9	99.7±41.4	0.006
VEGF at 3 rd month (pg/mL)*	67.2±31.1	61.6±29.9	76.8±31.4	0.063
G-CSF at admission (pg/mL)	10.6±5.9	10.9±6.5	10.0±4.2	0.494
G-CSF at 7th day (pg/mL)	14.3±7.3	13.5±7.6	15.7±6.5	0.192
G-CSF at 3 rd month (pg/mL)*	12.8±6.8	12.5±7.3	13.4±5.9	0.630
BDNF at admission (ng/mL)	3.3±0.9	3.2±0.9	3.4±0.9	0.490
BDNF at 7th day (ng/mL)	3.0±1.0	3.0±1.0	3.0±1.0	0.957
BDNF at 3 rd month (ng/mL)*	3.0±1.1	3.0±1.1	2.8±1.3	0.466

Results are expressed as mean± standard deviation, median [interquartile range] or n (percentage). * 3rd month serum factors were measured in 62 patients. P represents Student's T- test, Mann-U or Chi square for comparison among both groups of prestroke PhA

Table 2: Levels of angiogenic and neurogenic factors at each time point in patients with good (mRS≤2) and poor outcome (mRS >2) at 3rd month after stroke

	GOOD OUTCOME N=39	POOR OUTCOME N=44	p
VEGF at admission (pg/mL)	63.5±35.9	61.0±42.5	0.78
VEGF at 7th day (pg/mL)	100.4±39.8	65.1±37.0	<0.01
VEGF at 3rd month (pg/mL)*	80.5±37.9	52.1±24.0	<0.01
G-CSF at admission (pg/mL)	9.7±4.2	11.5±6.9	0.16
G-CSF at 7th day (pg/mL)	16.7±6.8	12.1±7.1	<0.01
G-CSF at 3rd month (pg/mL)*	14.1±7.8	11.2±4.8	0.09
BDNF at admission (ng/mL)	3.3±0.9	3.3±1.0	0.96
BDNF at 7th day (ng/mL)	3.0±1.0	2.9±1.0	0.69
BDNF at 3rd month (ng/mL)*	2.9±1.2	3.0±1.0	0.91

Results are expressed as mean± Standard Deviation. In the last column, p value for Student-t test comparisons. * 3rd month factors were measured in 62 patients.

Table 3: Multivariate analysis on predictors of good functional outcome at 3 months

	OR	95% CI
Pre-stroke PhA(for 1000 MET-min/week)*	2.29	1.61, 3.76
VEGF increment	1.06	1.03, 1.09

PhA: physical Activity. Results are expressed as Odds Ratio (OR) and 95% confidet intervals (CI)

Table 4: Multivariate analysis on predictors of final infarct volume*

	β	95% CI
Pre-stroke PhA*	-0.708	-0.950, -0.466
VEGF increment	-0.010	-0.019, 0.000
Baseline NIHSS	0.053	-0.008, 0.114
Age	-0.049	-0.079, -0.020

*Logarithmic transformation of IPAQ score and final infarct volume