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Psychological stress impairs ischemia-induced neovascularization: Protective effect of fluoxetine

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ABSTRACT

Background: Psychological stress (PS) has been associated with the development of cardiovascular diseases and adverse long-term outcomes after ischemic events. However, the precise mechanisms involved are not completely understood. Here we investigated the effect of PS on ischemia-induced neovascularization, and the potential therapeutic effect of fluoxetine in this condition.
Methods and results: Balb/c mice were subjected or not to chronic restraint stress. After 3 weeks, hindlimb ischemia was surgically induced by femoral artery removal. We found that blood flow recovery was significantly impaired in mice exposed to PS compared to controls (Doppler flow ratio (DFR) 0.61 ± 0.07 vs. 0.80 ± 0.07, $p < 0.05$). At the microvascular level, capillary density was significantly reduced in ischemic muscles of mice exposed to PS (38 ± 1 vs. 74 ± 3 capillaries per field, $p < 0.001$). This correlated with increased oxidative stress levels and reduced expression of VEGF and VEGF signalling molecules (p44/p42 MAPK, Akt) in ischemic muscles. We found that the number of pro-angiogenic cells (PACs) was significantly reduced in mice exposed to PS. In addition, oxidative stress levels (DCF-DA, DHE) were increased in PACs isolated from mice exposed to PS, and this was associated with impaired PAC functional activities (migration, adhesion, and integration into tubules). Importantly, treatment of mice exposed to PS with the selective serotonin reuptake inhibitor (SSRI) fluoxetine improved all the angiogenic parameters, and completely rescued PS-induced impairment of neovascularization.
Conclusion: PS impairs ischemia-induced neovascularization. Potential mechanisms involved include reduced activation of the VEGF pathway in ischemic tissues, increased oxidative stress levels and reduced number and functional activities of PACs. Our results suggest that fluoxetine may represent a novel therapeutic strategy to improve neovascularization and reduce ischemia in patients suffering from cardiovascular diseases and exposed to PS.

1. Introduction

Psychological stress (PS) is believed to be involved in both the development and the progression of cardiovascular diseases (CVD) [1,2]. For instance, PS led to a more than 2-fold increased risk of developing acute myocardial infarction (AMI) in the INTERHEART study [3]. Psychological distress has also been associated with poor outcome after percutaneous coronary interventions [4]. Interestingly, a recent study demonstrated that perceived psychological stress at the time of AMI is associated with worse long-term outcomes, even after adjustment for clinical and socio-demographic factors [5]. Patients exhibiting moderate or high stress levels had increased 2-year mortality compared with those having low levels of stress. Furthermore, psychological stress was independently associated with adverse 1-year health status, including a greater likelihood of suffering from residual angina [5].

The mechanisms underlying the association between PS and cardiovascular diseases are complex, involving both behavioural and physiological factors [2]. PS is associated with lifestyle changes (cigarette smoking, lack of exercise, obesity) that are well-established cardiovascular risk factors. In addition, PS is also associated with the development of deleterious physiological conditions including increased platelet aggregation [6], increased blood pressure [7], reduced insulin sensitivity [8], and impaired endothelial function [9]. The combination of these different factors could

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certainly contribute to explain the increased incidence of CVD associated with PS, and the higher mortality following cardiovascular events. However, another possibility that has not been tested so far is that PS could impair the reparative formation of new blood vessels in response to ischemia (i.e. neovascularization).

Neovascularization is one of the most important adaptive responses to ischemic vascular diseases [10]. This process involves the activation, proliferation and migration of mature endothelial cells (angiogenesis) [11]. Vascular endothelial growth factor (VEGF) has an essential role for the initiation and the maintenance of angiogenesis [12]. VEGF acts specifically on ECs via its receptors VEGFR1 (Flt-1), and VEGFR2 (Flk-1/KDR) to induce vascular permeability, cellular migration, cellular proliferation and tube formation [13]. The biological actions of VEGF have been associated with the induction of the MAP kinase pathway (EC proliferation) and the PI-3 kinase-Akt pathway (EC survival and migration) [13]. On the other hand, it has recently been demonstrated that postnatal neovascularization not only depends on the extension of the pre-existing vasculature, but also necessitates the action of bone marrow-derived pro-angiogenic cells (PACs) [14,15]. PACs have been shown to reach sites of neovascularization where they can differentiate into mature ECs and/or secrete angiogenic growth factors [16].

Neovascularization is very effective in experimental models using healthy animals and in young individuals. In these conditions, blood flow perfusion can usually be almost completely restored after ischemia. Atherosclerotic patients however are usually older and often exhibit different conditions and risk factors that might limit their ability to compensate for ischemia. The development of novel approaches to improve neovascularization and reduce tissue ischemia could have major clinical implications for these patients [17]. In the present study, we tested the hypothesis that PS impairs ischemia-induced neovascularization. Because selective serotonin reuptake inhibitors (SSRI) have been advocated as first-line treatment for anxiety and stress disorders [18], we also investigated the potential therapeutic effect of the SSRI fluoxetine on blood flow recuperation and neovascularization in the context of PS.

2. Methods

2.1. Murine ischemic hindlimb model and monitoring of blood flow

The protocol was approved by the Comité Institutionnel de Protection des Animaux (CIPA) of the Centre Hospitalier de l'Université de Montréal (CHUM). 5 to 7 week-old male BALB/c mice were purchased from Charles River (St. Constant, Canada). A total of 78 mice were used and 6 mice died following anaesthesia ($p = ns$ between groups). Mice were randomly grouped 4 mice per cage to establish a social structure before the experiment. Body weights were similar between groups at baseline (22 ± 1 g). Unilateral hindlimb ischemia was surgically induced after anesthesia with 2% isoflurane as previously described [19]. Hindlimb blood flow was monitored with a Laser Doppler perfusion imager (LDPI) system (Moor Instrument Ltd., Axminster, UK) after anesthesia with a ketamine-midazolam solution (100 mg/kg–5 mg/kg, intraperitoneally). Surgeries and Laser Doppler measurements were performed by a single observer blinded to the treatment group. To account for variables such as ambient light and temperature, the results are expressed as the ratio of perfusion in the left (ischemic) vs. right (non-ischemic) hindlimb [19]. Mice were killed at predetermined time points after surgery using an overdose of sodium pentobarbital. Blood samples were collected by cardiac puncture at the time of sacrifice.

2.2. Psychological stress (PS) model

Mice were maintained on a light/dark cycle of 12/12 h, on *ad libitum* chow with free access to water and under minimal stress conditions for at least 1 week before the beginning of the study. Restraint stress was induced using an established model [20]. Mice were placed in 50-ml conical centrifuge tubes with multiple ventilation holes, without penning the tail. Restraint was performed for 20 min in the morning, 5 days a week in order to avoid habituation. Mice were exposed to constraint stress beginning 3 weeks before the induction of hindlimb ischemia, and for 3 weeks after surgery. Constraint stress was not performed on days of LDPI measurements. Between stress sessions, mice remained in their home cages and had free access to food and water. Control unstressed mice were kept isolated from stress animals to avoid any acoustic or olfactory communication between the groups. To assess the behavioural and physical responses of mice to stress exposure, we used an observational stress severity score (SSS) [20]. Briefly, the standardized protocol is composed of five different parameters: urination, defecation, condition of fur, muscle tone, lethargy. The total SSS for each mouse consisted of points rated by the investigator based on the evaluation of each parameter. The total SSS ranges from 0 (no stress) to 33 (maximal stress) [20].

2.3. Drug treatment

Fluoxetine hydrochloride was obtained from commercially available 20 mg capsules (Prozac™; Eli Lilly Canada Inc., Toronto, Ontario) and diluted in mouse drinking water. The concentration of fluoxetine was determined from the average daily water consumption (x ml/mouse/day), and the average body weight per mouse (g/mouse) to achieve the desired dose. A dose of 18 mg/kg/day (160 mg/l) was chosen based on previous reports [21,22]. Fluoxetine solutions were protected from light in opaque water bottles and changed every 3 days. Control mice received regular drinking water. Fluoxetine treatment was started 3 weeks before the beginning of the stress sessions and maintained for the whole duration of the study.

2.4. Tissue preparation and immunohistochemistry

Whole ischemic hindlimbs were immediately fixed in tissue-fix overnight. After bones had been carefully removed, 3 μm thick tissue transverse sections of the hindlimbs were cut at the level of the gastrocnemius muscle and paraffin-embedded so that the whole leg could be analyzed on each section. Identification of endothelial cells was performed by immunostaining for platelet endothelial cells adhesion molecule-1 (PECAM-1 or CD31) with a rat monoclonal antibody directed against mouse CD31 (Pharmigen,San Diego, CA, USA), and capillaries were counted by a single observer blinded to the treatment regimen under a 20× objective and a 5× lens to determine the capillary density [19].

2.5. Flow cytometry analysis of pro-angiogenic cells (PACs)

The percentages of PACs contained in the total viable cell population derived from the spleen was measured by flow cytometry (FACSCalibur flow cytometer, Becton Dickenson, Oakville, Ontario, Canada) using the following fluorescence-coupled cell markers: CD34-FITC, VEGFR-2 (Flk1)-PE and CD117 (c-kit)-APC (eBioscience, CA, USA) [23]. Cell phenotypes were determined by the analysis of 300,000 events.