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Hypothesis related to the regulation of inducible nitric oxide synthase during carotid endarterectomy

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ABSTRACT

Sudden occlusion of an artery caused by a thrombus or emboli is the most frequent cause of acute brain ischemia (ABI). Carotid endarterectomy (CEA) represents the gold standard for preventing strokes of carotid origin. However, neuronal damage caused by ischemia and/or reperfusion may contribute to a poor clinical outcome after CEA. In response to shear stress caused by hypoxic-ischemic conditions in patients undergoing CEA, stimulation of the hypothalamic-pituitaryadrenal axis leads to biological responses known as hypermetabolic stress, characterized by hemodynamic, metabolic, inflammatory and immunological changes. These changes maintain homeostasis and assist recovery, but an unregulated inflammatory response could lead to further tissue damage and death of neurons. Nitric oxide (NO) is an important signaling molecule involved in several physiological and pathological processes, including ABI. However, an excess of NO could have detrimental effects. We hypothesized that the hypoxic-ischemic state induced by carotid clamping leads to overexpression of inducible NO synthase and that uncontrolled production of NO could adversely affect outcome after CEA.

Introduction

According to the World Health Organization, 15 million people/year experience a stroke [1]. The most common cause of stroke is acute brain ischemia (ABI) due to sudden occlusion of an artery caused by a thrombus or emboli (almost 85% of cases; the other 15% are hemorrhagic strokes) [2,3]. Carotid endarterectomy (CEA) is the gold standard for the treatment of carotid artery stenosis and preventing stroke of carotid etiology [4]. However, neuronal damage caused by ischemia and/or reperfusion may adversely affect outcome after CEA.

Increasing evidence suggests that overproduction of free radicals in the setting of ABI could induce functional and structural damage to neuronal cells [5]. During ABI, free radicals are generated by several mechanisms, including activation of inducible nitric oxide (NO) synthase (iNOS) [6–10]. NO is an important signaling molecule involved in several physiological and pathological processes [11] including ischemic brain injury [3]. The release of large amounts of cytokines during ABI recruits lymphocytes which infiltrate and accumulate in the damaged area [12]. By mediating the immune response, lymphocytes generate various inflammatory mediators, including iNOS [12]. Once expressed, iNOS can synthesize large amounts of NO [13]. This NO may have detrimental effects by producing highly reactive and toxic peroxynitrite when reactive oxygen species are present [3,14].

Hypothesis

Acute brain ischemia is a neurological condition during which various pathophysiological events could cause different neuronal damage [5]. In response to shear stress caused by hypoxic-ischemic conditions in patients undergoing CEA, stimulation of hypothalamic-pituitaryadrenal axis leads to biological responses known as hypermetabolic stress, characterized by hemodynamic, metabolic, inflammatory and immunological changes [15]. These changes maintain homeostasis and assist recovery. However, an unregulated inflammatory response could lead to further tissue damage and death of neurons. One of the potential mechanisms that promote neuronal damage related to ABI during surgery is an excess of NO [16]. We hypothesized that the hypoxic-ischemic state induced by carotid clamping leads to overexpression of iNOS and uncontrolled production of NO. This response could be responsible for complications and poor outcome

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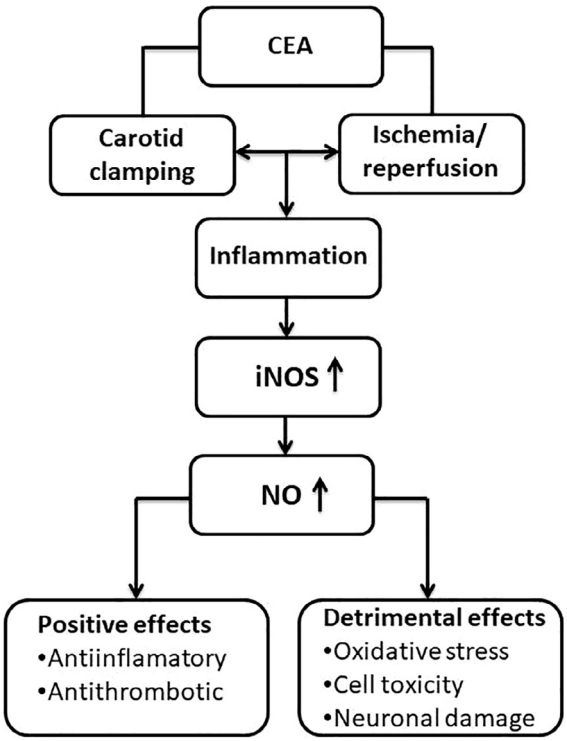

Fig. 1. The proposed mechanism of NO involvement in different complications and poor outcomes in patients undergoing CEA. CEA – carotid endarterectomy, iNOS – inducible nitric oxide synthase, NO – nitric oxide.

after CEA (Fig. 1).

Patients and methods

After written informed consent, 5 patients with carotid stenosis (> 70%) admitted for CEA were included in this pilot study. The study was conducted in accordance with the Declaration of Helsinki. The study protocol was approved by the Ethics Committee of Dedinje Cardiovascular Institute, Belgrade, Serbia (No. 4473). CEA was performed under general anesthesia. Blood was collected from the jugular vein of patients at 3 different times during CEA: 1 min before carotid cross-clamping (C1), 1 min after carotid cross-clamping (C2) and 1 min after reperfusion (C3). Jugular vein NO is ultimately converted into nitrite and nitrate which remain stable during cell culture and storage at −20 °C. NO production was measured by ion-selective electrodes to independently measure plasma nitrite and nitrate (Arrow STRAIGHT™ Lazar Research Laboratories, Inc, Los Angeles, USA) at the 3 specific time points.

The isolation of lymphocytes was started by adding 3 ml of LSM (Lymphocyte Separation Medium) in 10 ml tubes. Blood was diluted with saline (1:1), and 4–5 ml of diluted blood was added into each tube with LSM and then centrifuged for 30 min at 1500*g*. After centrifugation, the core layer with lymphocytes was extracted and transferred into tubes containing 1 ml of PBS. After shaking, the solution was washed twice with 1 ml of PBS. Supernatants were transferred into new tubes and stored at −70 °C. Cell viability was confirmed by the trypan blue exclusion method, and viability was > 90% [17].

The buffer for the protein isolation (pH 7.4) contains: 150 mM NaCl (sodium chloride), 20 mM tris (hydroxymethyl) aminomethane (TRIS), 2 mM EDTA (Ethylenediaminetetraacetic Acid), 2 mM DTT (Dithiothreitol), 1% non-ionic detergent Triton X-100, 10% glycerol, protease inhibitor cocktail (Complete Tablets ULTRA, Mini, EDTA-free, EASYPACK), phosphatase inhibitor cocktail (PhosSTOP), and 2 mM Na-orthovanadate, an inhibitor of protein phosphatase. After homogenization, the samples were sonicated twice for 5 s and lysed by incubation at +4 °C with rotation for 1 h. Lysed samples were then centrifuged for 30 min at 14000*g* at +4 °C. The supernatant was transferred into chilled tubes, and stored at −70 °C until further analysis.

The proteins (100 µg/lane) were separated by 10% or 12% SDS PAGE and transferred to PVDF membranes [18]. Membranes were blocked with 5% bovine serum albumin and probed with antibodies against iNOS (Abcam, Cambridge, UK), and actin (Santa Cruz Biotechnology Inc., CA, USA). After washing, the membranes were incubated with the appropriate secondary HRP – conjugated antibody and used for subsequent detection with the ECL reagent. In order to be sure that protein loading was equal in all samples, the blots were re-probed with the mouse anti-β-actin monoclonal antibody. The signals were quantified using ImageJ software (NIH, USA).

Results are presented as mean ± SEM. Statistical significance was evaluated by one-way ANOVA and Mann Whitney *U* test. The SPSS program for Windows (SPSS, Chicago, IL, USA) was used for statistical analyses. $p < 0.05$ was considered significant.

Results and discussion

NO synthesized by NOS has protective effects maintaining perfusion and providing anti-inflammatory, antithrombotic and antioxidant effects. However, carotid clamping may initiate overproduction of NO which may have harmful effects, such as autotoxicity and apoptosis [16]. Earlier studies show that NO mediates neurotoxicity in primary cortical cultures [19]. In addition, it has been reported that inhibition of NO synthesis reduced the complications caused by transient middle cerebral artery occlusion [20]. Increased production of free radicals during CEA, such as superoxide may react with NO and produce highly toxic peroxynitrite [21]. During middle cerebral artery occlusion and reperfusion in rats, increased NO and peroxynitrite formation in the circulation are followed with an increased expression of iNOS in vascular walls and the cortex [22]. In our study, in order to determine whether carotid clamping influences NO production, we measured plasma nitrite/nitrate concentration at 3 different times in patients undergoing CEA (Fig. 2). The concentration of NO is increased by 234% at C2 compared with C1 ($p < 0.05$). Furthermore, the concentration of plasma NO during C3 moment was additionally significantly elevated by 906%, compared with NO concentrations at C1 and C2 ($p < 0.05$ and $p < 0.001$, respectively).

To assess the effects of carotid clamping on NOS proteins expression

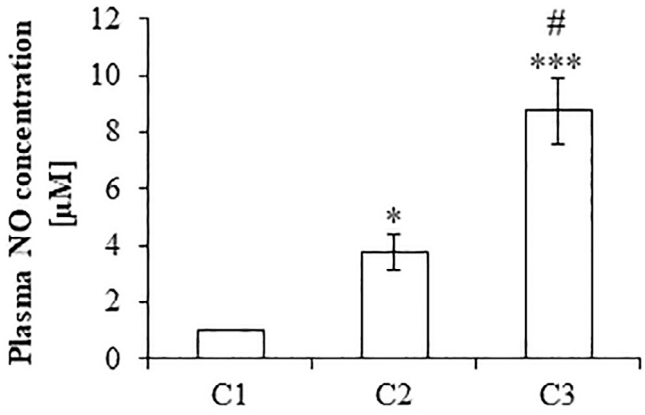

Fig. 2. The concentration of NO in plasma of patients at 3 different times during CEA. Plasma NO concentrations (n = 5). Results of C2 and C3 are expressed relative to value obtained for C1 measurement and represent mean ± SEM (C2, C3 vs. C1 $^{*}p < 0.05$, $^{***}p < 0.001$; C3 vs. C2 $^{\#}p < 0.05$). C1 – 1 min before carotid cross-clamping; C2 – during carotid cross-clamping; C3 – 1 min after reperfusion.

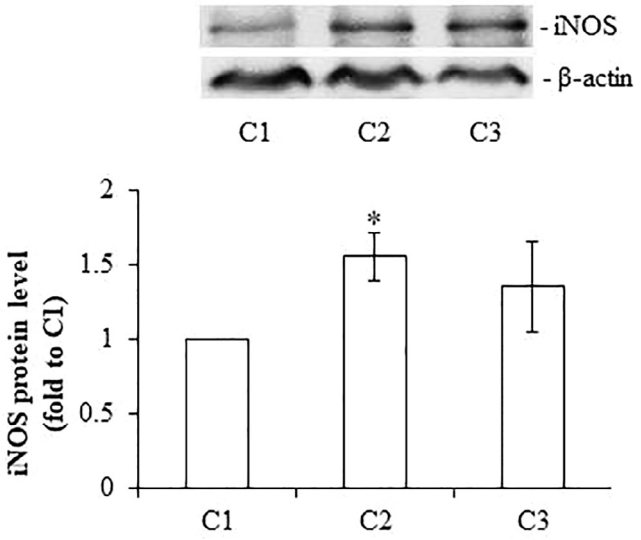

Fig. 3. The level of iNOS protein in lymphocytes of patients at 3 different times during CEA. Western blot analysis is showing relative iNOS protein levels (n = 3). Results of C2 and C3 are expressed relative to the value obtained for C1 measurement and represent mean ± SEM (C2 vs C1 $^{*}p < 0.05$). C1 – 1 min before carotid cross-clamping; C2 – during carotid cross-clamping; C3 – 1 min after reperfusion.

in lymphocytes, we examined levels of iNOS protein; this was increased by 22%, during C2 compared with C1 ($p < 0.05$) (Fig. 3). This suggests that the increase in iNOS protein in lymphocytes may contribute to the raised NO concentration in the circulation. Based on our results we suggest that clamping of the carotid artery and the development of inflammation or other pathophysiological stimuli may be responsible for this increase of iNOS protein level in lymphocytes.

Conclusions

Our preliminary result suggests that carotid clamping could lead to increased levels of plasma NO and iNOS protein in lymphocytes after CEA. The observed changes support our assumption that an increased NO level may be responsible for neuronal damage and poor outcomes in patients undergoing CEA. Thus, monitoring circulating NO levels during CEA may serve as an indicator of neuronal damage and stroke progress as well as help with the design of appropriate therapeutic measures [21,22]. Further research related to regulation of iNOS in pathophysiological conditions, such as ABI during CEA, is important to understand these processes and develop new therapeutic approaches to prevent and treat complications associated with CEA.

Conflict of interests

The authors confirm that this article content has no conflict of interest.

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