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Poly(ADP-ribose) polymerase is not involved in the neuroprotection exerted by azithromycin against ischemic stroke in mice

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ABSTRACT

Repurposing azithromycin has recently emerged as a promising strategy for the acute treatment of ischemic stroke. The mechanism of neuroprotection depends on the ability of this macrolide to promote polarization of microglia/macrophages towards beneficial M2 phenotypes. The immunomodulatory and anti-inflammatory effects of azithromycin, well documented in chronic inflammatory airway diseases, have been ascribed to the inhibition of the transcription factors nuclear factor (NF)-κB and activator protein (AP)−1. Since these inflammatory transcription factors are positively regulated by poly(ADP-ribose) polymerase (PARP)−1, an enzyme actively involved in ischemic brain injury, we have investigated whether the neuroprotective properties of azithromycin in ischemic stroke involve upstream modulation of PARP-1. Administration of a single dose of this macrolide antibiotic upon reperfusion reduced, to a similar extent in wild type and PARP-1 knockout mice, infarct brain damage produced by transient occlusion of the middle cerebral artery. Moreover, we demonstrated the lack of effects of azithromycin on PARP-dependent death of HeLa cells, as well as on activity of purified PARP-1 and PARP-2. Thus, azithromycin protects mice against ischemic stroke injury through a mechanism independent of PARP activation.

1. Introduction

Repurposing azithromycin has recently emerged as a promising strategy for the acute treatment of ischemic stroke (Amantea et al., 2016b). In fact, administration of a single dose of this macrolide up to 4.5 h after the insult significantly ameliorates histological and functional outcomes in mice subjected to middle cerebral artery occlusion (MCAo). The mechanism of neuroprotection depends on the ability of azithromycin to promote polarization of microglia/macrophages towards beneficial M2 phenotypes (Amantea et al., 2016b). The immunomodulatory and anti-inflammatory effects of azithromycin, well documented in chronic inflammatory airway diseases, have been ascribed to the inhibition of specific transcription factors, namely nuclear factor (NF)-κB and activator protein (AP)−1 (Bosnar et al., 2011; Parnham et al., 2014; Vrančić et al., 2012). Nevertheless, the upstream drug targets and their relevance in the setting of stroke have not been characterised.

Overactivation of poly(ADP-ribose) polymerase (PARP) has been reported to occur after brain ischemia and pharmacological inhibition of PARP-1 has emerged as a promising strategy to reduce post-ischemic brain damage (Gerace et al., 2015; Moroni and Chiarugi, 2009; Moroni, 2008). The deleterious effects of PARP include depletion of energetic pools, release of death signals from mitochondria, blood-brain barrier (BBB) rupture with consequent brain infiltration of immune cells, and induction of pro-inflammatory mediators (Gerace et al., 2015; Greco et al., 2014; Jagtap and Szabó, 2005). Since PARP-1 is a positive regulator of inflammatory transcription factors including NF-κB, AP-1 and nuclear factor of activated T-cells (NFAT), neuroprotection by PARP inhibitors has been ascribed to their anti-inflammatory properties (Chiarugi and Moskowitz, 2003; Haddad et al., 2006; Hamby et al., 2007). Interestingly, recent findings have highlighted that some antibiotics, most notably the neuroprotectant minocycline, exert anti-inflammatory effects by inhibiting PARP-1 (Alano et al., 2006; Banasik et al., 2012). This, along with the evidence that azithromycin shares downstream targets with PARP-1, supports our hypothesis that the neuroprotective and immunomodulatory properties of azithromycin in ischemic stroke may involve upstream modulation of PARP-1.

2. Materials and methods

2.1. Ethics statement

Animal care and experimental procedures were executed respecting the guidelines of the Italian Ministry of Health (DL 26/2014), in accordance with the European directive 2010/63/UE. The protocols (numbers 120000344, 1277/2015-PR and 272/2016-PR)

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were approved by the Committee set by the Ministry of Health at the National Institute of Health (Rome) and by the Institutional Animal Care and Use Committees. All efforts were made to reduce the number of animals used and their suffering and the study was conducted in compliance with the ARRIVE guidelines (Kilkenny et al., 2010).

2.2. Animals and drug treatment

Experiments were performed on C57Bl/6 male PARP-1 knockout (PARP-/-) and wild type (WT) mice (Harlan, Milan, Italy), weighting 24-26 g, obtained from homozygous breeding pairs. Animals were housed under controlled environmental conditions with ambient temperature of 22 °C, relative humidity of 65% and 12 h light:12 h dark cycle, with free access to food and water.

Mice were randomly allocated to each experimental group, namely drug or vehicle administration. Azithromycin (Zithromax®, azithromycin dihydrate for injection, Pfizer; 150 mg/kg) or vehicle (saline; 0.9% NaCl; 1 ml/kg) were injected i.p. upon reperfusion. The selection of the dose and time of drug administration was based on our previous observations (Amantea et al., 2016b).

2.3. Focal brain ischemia

Transient brain ischemia was induced in mice anaesthetised with isoflurane (1-2% in air) MCAo through insertion of a silicone-coated nylon filament (diameter: 0.23 mm, Doccol Corporation, Redlands, CA, USA) through the internal carotid artery (Amantea et al., 2011). During surgery, cerebral blood flow (CBF) was monitored over the cerebral cortex of the ischemic hemisphere, corresponding to the supply territory of the middle cerebral artery, by lased-Doppler flowmetry (Periflux System 5000, Perimed, Stockholm, Sweden). To this aim, a flexible laser-Doppler probe was glued onto the parietal bone and local CBF was continuously measured, keeping the animal under isoflurane anaesthesia. Animal displaying less than 70% CBF reduction following MCAo were excluded from the study. Thirty min after MCAo, the filament was withdrawn to allow reperfusion.

Cerebral infarct volume was was blindly determined 48 h after the beginning of reperfusion by staining with cresyl violet cryostat-cut 20 μm-thick coronal brain slices at 0.5 mm intervals from the frontal pole. Images of cresyl violet-stained sections were captured by a digital scanner and analysed using an image analysis software (ImageJ, version 1.30). Infarct volume (mm^3) was determined by summing the infarcted (pale) areas of the coronal tissue slices and multiplying the obtained value by the interval-thickness between sections.

2.4. Cell viability assay

HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine, 10% foetal bovine serum and antibiotics. Cultures were brought to 50-70% confluence and exposed for 1 h to 1-methyl-3-nitro-1-nitrosoguanidine (MNNG). After removal of MNNG, cells were incubated for 6 h with PJ34 (10 μM) or azithromycin (100 μM) and cell viability evaluated by measuring reduction of methylthiazolyl tetrazolium (MTT).

2.5. PARP-1 and PARP-2 activity assay

0.13 U of partially purified PARP-1 or PARP-2 (Trevigen, USA) were incubated at 37 °C in a final volume of 100 μL of 20 mM Tris-HCl buffer (pH 8), containing 20 mM $MgCl_2$, 5 mM DL-dithiothreitol, 20 μg sonicated calf thymus DNA (Invitrogen, San Diego, CA, USA), 0.5 mg/mL bovine albumin and 1 μL [adenine-2,8-^{3}H]-NAD (35.5 nmol, Perkin Elmer, Milan, Italy). The PARP inhibitor PJ34 (10 μM, Sigma Aldrich, Milan, Italy) or azithromycin (100 μM, Zitromax, Pfizer) were added to the enzymatic reaction to test their effect on PARP activity. The mixture was incubated at 37 °C for 1 h and the reaction terminated by adding 0.5 ml of 50% trichloroacetic acid followed by brief centrifugation. After two gentle washes of the pellet with 1 ml distilled water, the radioactivity incorporated from [adenine-2,8-^{3}H]-NAD into proteins was evaluated by liquid scintillation spectrometry (Tri-Carb 1900 TR; Packard, Meriden, CT, USA).

2.6. Statistical analysis

Data were expressed as mean ± S.E.M. and analysed by one- or two-way ANOVA as indicated in figure legends. P values below 0.05 were considered statistically significant.

3. Results

In order to investigate the putative involvement of PARP-1 in the neuroprotective effects of azithromycin, we evaluated the effect of this drug on brain damage produced by transient MCAo in PARP-1 null mice. Deletion of PARP-1 resulted in reduced infarct damage after transient MCAo (Fig. 1A-C), whereby CBF reduction was not affected (Fig. 1D). Administration of a single dose of azithromycin (150 mg/kg, i.p.) upon reperfusion significantly reduced brain damage in both WT and PARP-/- mice, suggesting that PARP-1 is not involved in the mechanisms of neuroprotection exerted by this drug. Moreover, the beneficial effects of azithromycin were not associated with modifications of cerebral perfusion, since the reduction of CBF induced by MCAo was unaffected by administration of a neuroprotective dose of this drug (Fig. 1D).

To confirm the lack of involvement of PARP in the mechanisms of neuroprotection exerted by azithromycin, we also evaluated PARP activity *in vitro*. Azithromycin (100 μM for 6 h) did not affect HeLa cells viability, neither this treatment had effect on cell death induced by the PARP-1-activating compound MNNG (Fig. 2A). By contrast, the PARP inhibitor PJ34 (10 μM for 6 h) abolished cell death produced by MNNG (Fig. 2A). In fact, PJ34 is a potent inhibitor of recombinant PARP-1 and PARP-2; whereas, azithromycin did not show any effect on the activity of these enzymes (Fig. 2B).

4. Discussion

In the present study, we have demonstrated that azithromycin protects mice against ischemic stroke injury through a mechanism independent of PARP activation. In fact, administration of a single dose of this macrolide antibiotic upon reperfusion reduced to a similar extent infarct brain damage in WT and PARP-/- mice. To further confirm this conclusion, we demonstrated the lack of effects of azithromycin on PARP activity induced by MNNG in HeLa cells and on purified PARP-1 and PARP-2.

Azithromycin is a macrolide antibiotic that, in addition to its prolonged antibacterial effect, is endowed with immunomodulatory and anti-inflammatory properties (Parnham et al., 2014). We have previously demonstrated that this drug represents a promising candidate to be developed for ischemic stroke treatment. The improvement of histological and functional outcomes produced by a single administration of azithromycin to mice subjected to transient MCAo involves modulation of the immune system (Amantea et al., 2016b). In particular, azithromycin is able to promote polarization of microglia and peripheral macrophages towards the 'beneficial' M2 phenotype (Amantea et al., 2016b). This is consistent with the evidence that this drug shifts both mouse and human macrophages from the classically activated M1 to the alternatively activated M2 phenotype through inhibition of the transcription factors NF-κB and