Role of RIP3 in PDT-induced glioblastoma Cell Death

Grégory Fettweis, Isabelle Coupienne, Marianne Fillet, and Jacques Piette
Virology and Immunology Unit, GIGA-R, University of Liège, Belgium

Introduction

Glioblastoma are the deadliest type of brain cancer. They are associated with poor survival and a high degree of recurrence despite removal by surgical resection and treatment with chemo and radiotherapy. 5-aminolevulinic acid (5-ALA)-based photodynamic therapy (PDT) was recently shown to sensitize human glioblastoma cells (LN18) to programmed necrosis, also called necroptosis. RIP3 (Receptor Interacting Protein 3) kinase, a key factor of the necroptotic signaling pathway, is clearly implicated in PDT-induced glioblastoma cell death. It was shown to associate with RIP1 kinase in a protein complex called necrosome, where it autophosphorylates and allows the downstream necroptotic events to take place. Intriguingly, the other factors commonly present in the necrosome, namely Caspase-8 and FADD were not encountered in PDT-induced pro-necrotic complex. In order to caracterise the member of the PDT-induced complex, we transduce LN18 cells with tagged-RIP3 construction and analyses RIP3 immunoprecipitate by mass spectrometry. This approach gave us a list of proteins potentially associated with RIP3 after PDT. Among those, we confirmed the interaction of 14-3-3-ε with RIP3. Further experiments allowed us to show the presence of 14-3-3-ζ and TSC2 in the complex both in cells overexpressing RIP3 and in an endogenous model.

1. Glioblastoma cells die by an atypical RIP3-dependent necrosome after 5-ALA-PDT

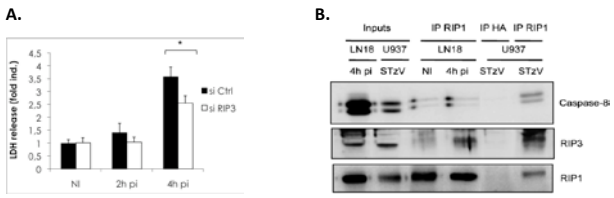


Fig. 1.A. Depletion of RIP3 by siRNA leads to a decreased necrosis induction in response to PDT in LN18 cells (as shown by Lactated DeHydrogenase release assay). pi = time post-irradiation *p < 0.05). **Fig. 1.B.** Analysis of the necrosome complex components shows that usually present proteins as FADD and Caspase-8 do not take part in PDT-induced pro-necrotic complex. U937 treated with smac mimetic, Staurosporine and zVAD-fmk (STzV), are used as a positive control for "canonical" necrosome formation. pi = time post-irradiation

2. Investigation of RIP3 partners by a Proteomic approach

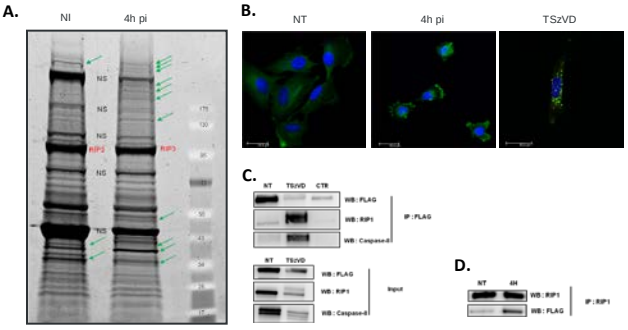


Fig. 2.A. LN18 human glioblastoma cells were transduced with FLAG-RIP3-eGFP and 60 mg of total lysate were pre-cleared for 4H at 4° C and then incubated with anti-FLAG coupled-beads over night at 4° C. Beads were washed with 50 mL of a 400mM NaCl buffer and then eluated with 2mL of 200 µg/mL 3X FLAG peptide. Proteins were concentrated and loaded on a 4-12% gel. Proteins were stained with Sypro ruby, bands cut and analyzed by Mass spectrometry. (NI= non irradiated, pi= time post-irradiation). **Fig. 2.B.** Immunoflurescence showing the clustering of 3XFLAG RIP3-eGFP after 5-ALA-PDT and TNF-α/Smac-mimetic/zVAD-fmk treatment (8H) .(NT= non irradiated, pi= time post-irradiation, TSzVD = TNF-α/Smac-mimetic/zVAD-fmk). **Fig. 2.C.** Validation of the 3XFLAG-RIP3-eGFP construct by FLAG immunoprecipitation under TNF-α/Smac-mimetic/zVAD-fmk treatment (8H) showing a complex containing FLAG-RIP3-eGFP, the Caspase-8 and RIP1 (NT= non irradiated, TSzVD = TNF-α/Smac-mimetic/zVAD-fmk, CTR = control). **Fig. 2.D.** FLAG-RIP3-eGFP and RIP1 forms a complex after PDT treatment here showed by an immunoprecipitation of RIP1. (NT = non irradiated, 4H = 4H post-irradiation).

3. Formation of a Flag-RIP3-eGFP/TSC2/14-3-3 complex after PDT treatment

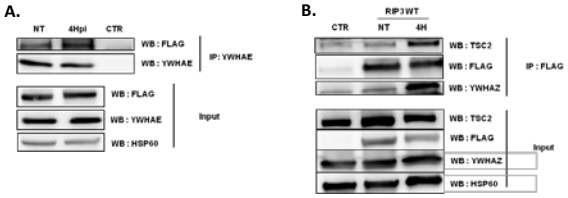


Fig. 3.A Immunoprecipitation of YWHAE shows its interaction with FLAG-RIP3-eGFP 4 hours after of 5-ALA-PDT treatment. (NT= non irradiated, pi= time post-irradiation, CTR = control). **Fig. 3.B.** FLAG Immunoprecipitation reveals interaction between TSC2, YWHAZ and FLAG-RIP3-eGFP 4 hours after 5-ALA-PDT treatment. (NT= non irradiated, 4H= 4H post-irradiation, CTR = LN18 transduced with eGFP)

4. RIP3 KD or RHIM domain mutations do not affect the TSC2/RIP3/14-3-3 complex formation

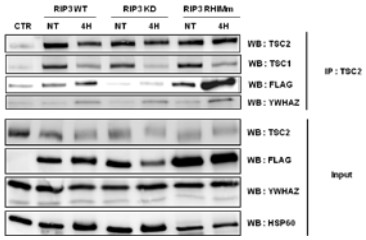


Fig. 4. Immunoprecipitation of TSC2 shows its interaction with FLAG-RIP3-eGFP and YWHAZ 4 hours after of 5-ALA-PDT treatment independtly of RIP3 Kinase Dead or RHIM domain mutations. (NT= non irradiated, 4H = 4H post-irradiation, CTR = LN18 transduced with eGFP, KD = Kinase Dead, RHIM = Rip Homology Interaction Motif)

5. TSC2/RIP3/14-3-3 complex forms endogenously after PDT treatment

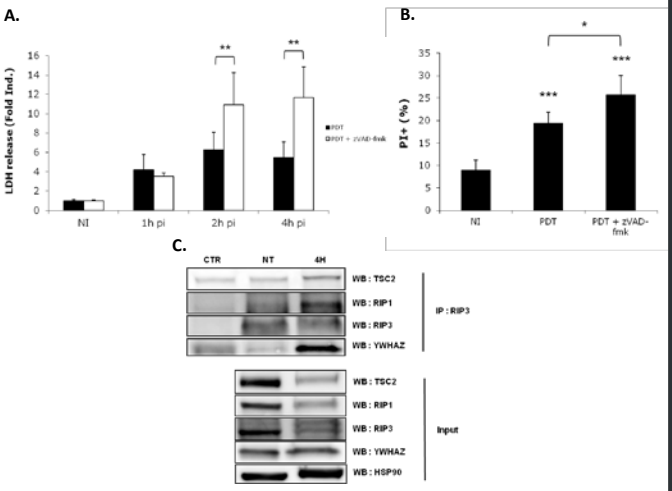
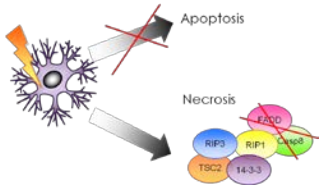


Fig.5.A-B. F98 Rat glioma cells died by necroptosis and expressed RIP3. **A.** In F98 rat glioma cell line, 5-ALA-PDT treatment induces necrosis which is increased in the presence of zVAD-fmk (as shown by Lactated DeHydrogenase release assay). (NI = non-irradiated, pi = time post-irradiation **p < 0.01). **B.** Propidium Iodide positive F98 cell population analyzed by flow cytometry increases 4 hours after 5-ALA-PDT treatment, wich is reinforced in the presence of zVAD-fmk. (NI = non-irradiated, *p<0,05, ***p < 0.001). **Fig.5.C.** RIP3 interacts with TSC2, YAWHZ and RIP1 4 hours after 5-ALA-PDT treatment as showed by the immunoprecipitation of endogenous RIP3. (CTR = control, NT = non irradiated, 4H = 4H post-irradiation).

5. Conclusions



Contact : gfettweis@ulg.ac.be