VI Seminario Nazionale per Dottorandi in Farmacologia e Scienze Affini
Siena, Certosa di Pontignano, 23 - 26 Settembre 2002

NEUROPATHIC PAIN IN RAT: A BIOMOLECULAR APPROACH

Siniscalco D., 2° anno di corso del Dottorato di Ricerca in Scienze Farmacologiche e Fisiopatologia Respiratoria, ciclo XVI. Durata del Dottorato in anni: 4. Sede di servizio: Department of Experimental Medicine, section of Pharmacology "L. Donatelli". Faculty of Medicine and Surgery, Second University of Naples. Naples.

Introduction Pathological pain syndromes, often referred to as neurophatic pain, are characterized by hyperalgesia and allodynia. Although previous morphological studies (Whiteside and Munglani, 2001) have suggested the occurrence of programmed cell death in the spinal cord following peripheral nerve injury, there is no molecular study aimed at investigating whether or not changes in the expression of apoptotic genes may occur in the development neurophatic pain syndrome.
This study uses a biomolecular approach to asses the occurrence of apoptosis in the rat spinal cord by 1, 3 and 7 days sciatic nerve chronic constrictive injury (CCI). Moreover, the effect of MPEP, a selective mGlu5 receptors antagonist, was evaluated on molecular changes induced by CCI.
Material and methods Total RNA was extracted from homogenized whole lumbar L4-L5 spinal cord using an RNA Tri-Reagent. The mRNA levels of the genes under analysis were measured by RT-PCR amplification. The measured mRNA levels were normalised with respect to HPRT, chosen as housekeeping gene, and the gene expression values were expressed as arbitrary units ± SE. For the protein extraction, the spinal cord was minced into small pieces with a blender in denaturating lysis buffer. The tissue was sonicated in ice. Each sample was loaded, electrophoresed in a 8% polyacrylamide gel, electroblotted onto a nitrocellulose membrane and incubated with primary antibodies to detect p53 or E2F1. Cathepsin D activity was determined as described by Kagedal with some modification. In short, 100 µg of total protein of spinal cord was mixed with hemoglobin 4%. The solution was incubated for 60 min at 45°C. The reaction was terminated by adding 3% trichloric acid at 4°C for 30 min. The amount of peptides released was estimated by the method described by Bradford. Cathepsin D activity was expressed as Absorbance Units per milligram of total protein ± SE.
Results The semiquantitative analysis of mRNA levels measured by RT-PCR amplification, at the whole L4-L5 lumbar spinal cord of rats, showed a no significant increase in the pro-apoptotic *bax* gene together with a slight decrease in the anti-apoptotic *bcl-2* gene (table 1). However, the *bax*/*bcl-2* ratio resulted to be increased by 80% (mean ± SE of arbitrary units: 0.43 ± 0.08 *vs* 0.24 ± 0.08 in ligated and sham rats, respectively) by 1 day sciatic nerve CCI. A massive increase by 370% (mean ± SE of arbitrary units: 0.85 ± 0.10 *vs* 0.18 ± 0.09 in ligated and sham rats, respectively) of this ratio was observed by 3 days CCI. In particular, the pro-apoptotic *bax* gene was dramatically over-expressed up to 320% at this time as compared to the sham rats (table 1). Seven days post-CCI, no significant change in the *bax*/*bcl-2* ratio was observed (mean ± SE of arbitrary units: 0.14 ± 0.02 *vs* 0.13 ± 0.01 in ligated and sham rats, respectively), due to an increased expression (up to 60%) in the anti-apoptotic *bcl-2* gene and a decreased expression (up to 40%) in the pro-apoptotic *bax* gene (table 1). In rats treated with MPEP (2 mg/Kg i.p. twice, daily), a selective mGlu5 receptors antagonist, *bax*/*bcl-2* ratio showed no significant change by 3 days CCI (mean ± SE of arbitrary units: 0.22 ± 0.08 *vs* 0.18 ± 0.09 in MPEP-treated and sham rats, respectively) and 7 days CCI (mean ± SE of arbitrary units: 0.12 ± 0.03 *vs* 0.13 ± 0.01 in MPEP-treated and sham rats, respectively). MPEP treatment increased up to 33% the anti-apoptotic *bcl-2* gene expression (table 1), whereas the mRNA levels of the pro-apoptotic *bax* gene were dramatically reduced if compared to 3 days CCI untreated rats. Although the pro-apoptotic *bcl-xS* gene expression resulted to be decreased significantly by 1 day CCI, the *bcl-xS*/*bcl-xL* ratio, however, was not changed (mean ± SE of arbitrary units: 0.20 ± 0.03 *vs* 0.24 ± 0.04 in ligated and sham rats, respectively). The *bcl-xS*/*bcl-xL* ratio was increased by 22% by 3 days CCI (mean ± SE of arbitrary units: 0.28 ± 0.03 *vs* 0.23 ± 0.09 in ligated and sham rats, respectively), and decreased by 23% (mean ± SE of arbitrary units: 0.20 ± 0.04 *vs* 0.26 ± 0.03 in ligated and sham rats, respectively) by 7 day CCI (table 1). In rats treated with MPEP (2 mg/Kg i.p. twice, daily) the *bcl-xS*/*bcl-xL* ratio was not changed either 3 days (mean ± SE of arbitrary units: 0.18 ± 0.08 *vs* 0.23 ± 0.09 in MPEP-treated and sham rats, respectively) and 7 days CCI (mean ± SE of arbitrary units: 0.20 ± 0.05 *vs* 0.26 ± 0.03 in MPEP-treated and sham rats, respectively). MPEP treated increased the mRNA levels of the anti-apoptotic *bcl-xL* gene up to 60% and 150% as compared to the sham operated rats by 3 and 7days CCI, respectively.
To check the presence of gliosis, changes in the expression of *GFAP* and *nestin* genes were analysed. The mRNA levels of the *GFAP* gene, measured by semiquantitative RT-PCR amplification and normalized to the signal obtained for the housekeeping gene *HPRT*, were increased by 3 and 7 days post-CCI (table 1). Similarly the mRNA level of the *nestin* increased 1, 3 and 7 days post-CCI. In rats treated with MPEP (2 mg/Kg i.p. twice, daily) the mRNA level of the *GFAP* gene were not modified as compared to CCI and untreated rats (table 1). MPEP treatment reduced the mRNA levels of

the *nestin* gene in CCI MPEP-treated rats is reduced up to 121% *vs* the sham operated rats by 3 days CCI and not changed as compared to CCI and untreated rats by 7 days CCI, respectively.
To check further the occurrence of apoptosis, the expression of the p53 and E2F1 transcription factors were analysed. The Western blot analysis with anti-P53 and anti-E2F1 antibodies showed that these transcriptional factors were never modified at all times in all groups of rats (data no shown).
We also excluded the activation of an apoptosis -like pathway *via* non caspase lysosomal aspartic protease Cathepsin D. Also in this case, the Cathepsin D activity was not modified in the groups of rats (mean ± SE of Absorbance Units/mg total protein: 0.85±0.10 and 0.81±0.15 in naive and sham rats, respectively; mean ± SE of Absorbance Units/mg total protein: 0.70±0.14, 0.76±0.11, 0.72±0.10 by 1, 3 and 7 days CCI rats, respectively).
Conclusion In conclusion, this study shows that: a) neuropathic pain may be triggered and maintained by glutamate-induced apoptosis in the spinal cord; b) a p53-independent apoptotic pathway seems to be activated and the expression of the pro-apoptotic genes shows a maximum by 3 days CCI, with contemporary gliosis; c) in the chronic phase of pain there is activation of the anti-apoptotic genes; d) MPEP treatment increases the anti-apoptotic gene expression, indicating a neuroprotective role for this mGlu5 receptors antagonist.

Table 1. Semiquantitative analysis of pro-apoptotic (*bax* and *bcl-xS*) and anti-apoptotic (*bcl-2* and *bcl-xL*) genes and structural glial proteins (*nestin* and *GFAP*) mRNA levels.

gene	N	L1	S1	L3	S3	L7	S7	MPEP 3	MPEP 7
bax/HPRT	0.19±0.03	0.33±0.06	0.17±0.04	**0.76±0.09***	0.18±0.02	0.24±0.03	0.14±0.01	0.28±0.05	0.22±0.08
bcl-2/HPRT	1.06±0.05	0.76±0.03	0.71±0.07	0.89±0.1	0.97±0.09	**1.75±0.28***	1.08±0.15	**1.29±0.08***	**1.77±0.21***
bcl-xS/HPRT	0.70±0.05	**0.43±0.04***	0.65±0.19	0.74±0.07	0.50±0.28	0.63±0.03	0.37±0.25	0.62±0.06	0.74±0.04
bcl-xL/HPRT	2.70±0.20	2.12±0.24	2.68±0.08	2.60±0.58	2.20±0.02	**3.10±0.09***	1.47±0.56	**3.54±0.11***	**3.67±0.16***
nestin/HPRT	0.82±0.04	**2.05±0.1***	0.75±0.01	**3.40±0.35***	0.96±0.19	**1.80±0.12***	1.07±0.05	**2.13±0.02***	1.21±0.09
GFAP/HPRT	5.49±0.49	**7.14±0.39***	4.40±0.32	**13.74±0.66***	5.64±0.60	**11.63±0.70***	5.08±0.47	**13.9±0.6***	**14.4±0.5***

The numbers show the mRNA levels (mean± SE) of the genes under analysis measured by RT-PCR amplification. Numbers in the first row of the table indicate the days (1, 3, 7) post surgery in naïve (N), ligated (L), sham (S) and twice-daily treated rats with MPEP (2 mg/Kg i.p.), a selective antagonist of mGlu5 receptors. *$p<0.05$ *vs* naïve rats (bold); as analysed by ANOVA, followed by Student-Neuman-Keuls test.

References

1. Merry DE and Korsmeyer SJ. (1997) Annu. Rev. Neurosci. 20: 245-267.

2. Whiteside GT and Munglani R. (2001) J. Neurosci. Res. 64: 168-173.

3. Maione S, Siniscalco D, Galderisi U, de Novellis V, Uliano R, Di Bernardo G, Berrino L, Cascino A and Rossi F. (2002) Neuroreport 13(1): 101-106.