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Modulation of brain apoptosis-related proteins by the opioid antagonist naltrexone in mice

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Abstract

Neuronal loss by apoptosis has been implicated in some neural pathologic disorders. Increasing evidence suggests a neuroprotective effect for opioid antagonists, such as naloxone and naltrexone, in a variety of neural damage experimental models and in the clinic. The purpose of the present study was to analyse the effects of naltrexone on the expression levels of proteins regulating the extrinsic (FasL and Fas) and the mitochondrial (Bcl-2, Bcl-x_L, Bad and Bax) apoptotic pathways, as well as the active fragment of the executioner caspase-3 in the mouse brain. Western blotting showed that a single injection of naltrexone (1 mg/kg) induced a down-regulation of the pro-apototic proteins Fas, FasL, Bad and Bax. Our results suggest that naltrexone provides neuronal protection against injuries activating either mitochondrial, or death receptor-apoptotic pathways.

Keywords: Naltrexone; Opioid antagonist; Neuroprotection; Bcl-2 family; Fas signalling; Apoptosis

Apoptosis, or programmed cell death, can be triggered by signals arising from within the cell or by extrinsic death activators though the activation of a cascade of proteolytic enzymes termed caspases. The intrinsic apoptotic pathway involves the release from the mitochondria into the cytosol of cytochrome *c* and other apoptogenic proteins [13]. This apoptotic pathway is regulated by the Bcl-2 family of proteins integrated by anti-apoptotic (Bcl-2, Bcl-x_L, etc.) and pro-apoptotic (BH3 only family and Bax family) members [7]. The extrinsic apoptotic pathway is mediated by the tumor necrosis factor receptor (TNF-R) superfamily of death receptors. Fas (CD95 or APO-1) is a prototypical member of this family, whose binding with cytokines belonging to the TNF family, such as Fas ligand (FasL), induces cell death through caspase activation [2]. Neuronal loss by apoptosis has been implicated in some pathological conditions such as neurodegenerative disorders, acute and chronic neural damage processes, exposure to neurotoxic agents, etc. [24].

Numerous studies indicate that activation of opioid receptors by agonist drugs or by endogenous opioid peptides promote apoptosis in a variety of human and rodent cell preparations in vitro, including neuronal and microglial cultured cells [28]. Some of these studies suggest the involvement of the FasL/Fas apoptotic pathway and caspase activation in opioid induced apoptosis [26,30]. Studies performed in rodents indicate that chronic exposure to opioid drugs produce cell death in the central nervous system by activating both the mitochondrial and the Fas/FasL-mediated apoptotic pathways [3,8,10,19]. On the other hand, recent studies have described that the non-selective antagonist of opioid receptors, naloxone, exerts a neuroprotective effect against degeneration induced by inflammatory stimuli and by β-amiloid in neuron-glia cultures, through the inhibition of TNFα release by microglia [15,16–18,22]. Furthermore, in vivo, naloxone also exerts a neuroprotective effect against cerebral ischemic injury in rats, through a not well-understood mechanism [4,5,15]. Indeed, the knowledge of the mechanisms involved in the neuroprotective effect of opioid antagonists is of paramount therapeutic importance. Here, we have analyzed the effects of the long-acting naloxone analogue, naltrexone, on the expression levels of proteins regulating the extrinsic (FasL and Fas) and the mitochondrial (Bcl-2, Bcl-x_L, Bax, Bad) apoptotic pathways, as well as the executioner caspase-3 in the mouse brain.

Male C57BL/6J mice were used. They were housed in a room kept at 22 °C with a 12-h light:12-h dark cycle. Food and

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water were provided ad libitum. This study was approved by the Cantabria University Institutional Laboratory Animal Care and Use Committee and was carried out in accordance with the Declaration of Helsinki, and the European Communities Council Directive (86/609/EEC).

For acute treatments, mice received a single intraperitoneal injection of the opioid antagonist naltrexone (1 mg/kg) or saline (0.1 ml), and they were killed after 6 h, by using a pentobarbital overdose.

Brains were removed, and the cerebral cortices were dissected on ice and homogenized in 10 mM Tris–HCl, 1 mM EDTA buffer, containing 2% sodium dodecylsulfate (SDS) and protease inhibitors [4-(2 aminoethyl) benzenesulfonyl fluoride hidrochloride 1.3 mM; leupeptin 10 μg/ml; pepstatin A 10 μg/ml; E-64 5 μg/ml; antipain 10 μg/ml]. Lysates were boiled 10 min, sonicated, and then centrifuged at 5000 × *g* for 5 min. The supernatant fluid is the total cell lysate. Identical amounts of whole-cell protein from each sample were loaded on sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), electrophoresed, and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). The following antibodies were used: monoclonal anti-FasL (1:1500; abcam), monoclonal anti-Fas (1:5000; Transduction Labs, Lexington, KY, USA), monoclonal anti-Bcl-2 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), monoclonal anti-Bcl-x_L (1:500; Transduction Labs), monoclonal anti-Bad (1:500; Transduction Labs), monoclonal anti-Bax (1:100; Santa Cruz Biotechnology), and polyclonal anti-caspase-3 active (1:200; abcam). The blots were incubated with the appropriate peroxidase-labeled secondary antibody for 1 h at room temperature. Immunoreactivity was detected with an enhanced chemiluminescence detection system (ECL advanced, Amersham-Biosciences, Arlington Heights, IL, USA) and visualized by Amersham Hyperfilm-ECL. The film was scanned, and the integrated optical density (OD) of the bands was estimated (Scion Image software, Scion Corporation, Frederick, MD, USA). Relative variations between the bands of the problem and the control samples were calculated in the same film. Each individual sample was measured by duplicate in at least three independent experiments. Measurements were in the linear range. To ensure equal loading, the blots were reprobed with a mouse monoclonal anti α-tubulin antibody (1:1000; Sigma).

The results are expressed as mean ± S.E. from five individuals per group. Statistical analysis was carried out by the Student's *t*-test (GraphPAD InStat software). $P < 0.05$ was considered to be statistically significant.

In western blot immunoassays, the different proteins were detected in bands located at ~35 kDa for native Fas, ~33 kDa for FasL, ~26 kDa for Bcl-2, ~26 kDa for Bcl-x_L, ~30 kDa for Bad, ~21 kDa for Bax, ~17 kDa for active caspase-3 and ~50 kDa for α-tubulin. Actual integrated optical density values of representative films from each experimental group are presented in Table 1.

As shown in Fig. 1, 6 h after a single injection of naltrexone to mice ($n = 5$), a significant down-regulation of FasL (32.4 ± 4.1%, $P < 0.001$) and the native form of Fas (64.9 ± 12.0%, $P < 0.01$) was evidenced in the brain cortex. The expression levels of the anti-apoptotic proteins belonging to the Bcl-2

Table 1
Integrated optical density (IOD) values scanned from a representative film of each antibody tested

	Control	Naltrexone
Fas	1299.6 ± 42.4	906.6 ± 37.1
FasL	505.9 ± 69.8	173.1 ± 19.8
Bcl-2	938.1 ± 25.1	995.7 ± 213.9
Bcl-x_L	1368.9 ± 83.5	1467.4 ± 67.5
Bax	1803.4 ± 51.6	1357.9 ± 27.1
Bad	850.8 ± 42.0	505.5 ± 83.6
Active saspase-3	496.6 ± 39.0	434.8 ± 33.8

Data are expressed as mean IOD values ± S.E. from five animals of each group. The IOD of the bands was estimated with the Scion Image software.

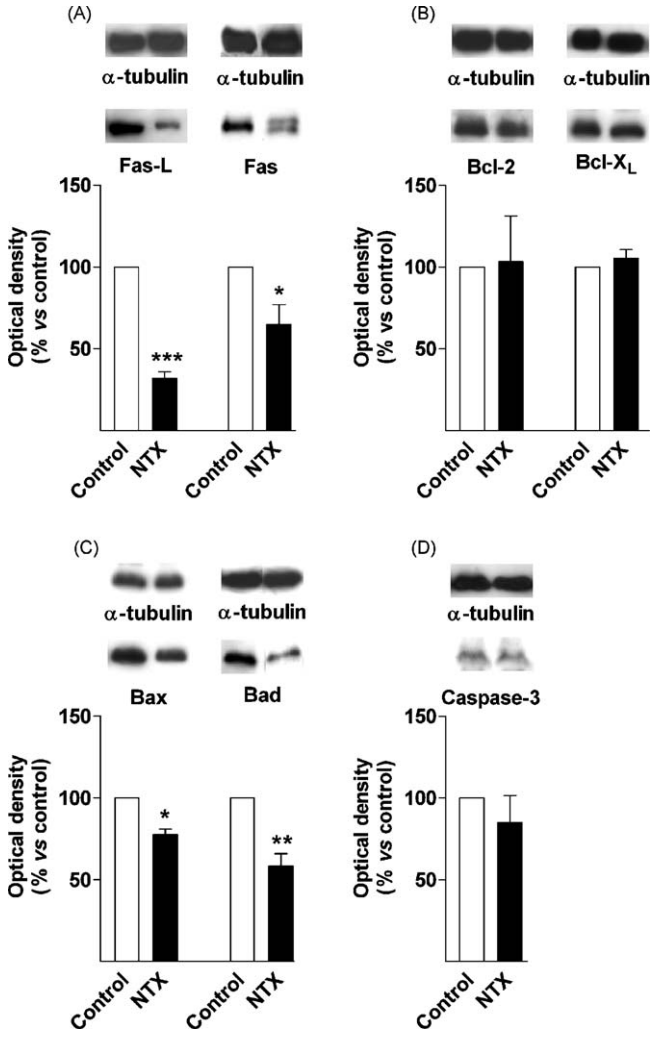

Fig. 1. Western blot analysis of Fas-L and Fas (**A**); Bax and Bad (**B**); Bcl-2 and Bcl-X_L (**C**); and active caspase-3 (**D**) immunoreactivity levels in cortices from mice treated with an acute dose of naltrexone (1 mg/kg, i.p.). Blots were digitized, and the integrated optical density was estimated using densitometric analysis (Scion Image). Immunoreactivity changes of naltrexone-treated animals were expressed relative to the values of saline-treated mice (defined as the 100% value). Each individual sample was measured in duplicate in at least three independent experiments. Data are mean ± S.E.M. from five animals. Statistical analysis was carried out by the Student's *t*-test; $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Representative immunoblots directly scanned from the films are shown.