Biochemical and Biophysical Research Communications 391 (2010) 426–430

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Toll-like receptor 2 is required for opioids-induced neuronal apoptosis

Yi Li [a,b,1], Hui Li [c,a,1], Yi Zhang [a], Xiuli Sun [a], Gregory A. Hanley [d], Gene LeSage [a], Ying Zhang [e], Shenggang Sun [c], Ying Peng [b,*], Deling Yin [a,*]

[a] Department of Internal Medicine, College of Medicine, East Tennessee State University, Johnson City, TN 37614, USA
[b] Department of Neurology, The Second Affiliated Hospital, Sun Yat-sen University, Guangzhou 510120, China
[c] Department of Neurology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China
[d] Department of Laboratory Animal Resources, College of Medicine, East Tennessee State University, Johnson City, TN 37614, USA
[e] Department of Molecular Microbiology and Immunology, Johns Hopkins University, Baltimore, MD 21205, USA

ARTICLE INFO

Article history:
Received 6 November 2009
Available online 13 November 2009

Keywords:
TLR2
Morphine
Apoptosis
Neurons

ABSTRACT

Toll-like receptor 2 (TLR2), a key immune receptor in the TLR family, is widely expressed in various systems, including the immune and nervous systems and plays a critical role in controlling innate and adaptive immune responses. We previously reported that opioids inhibit cell growth and trigger apoptosis. However, the underlying mechanism by which TLR2 mediates apoptosis in response to opioids is not yet known. Here we show that chronic morphine treatment in primary neurons dramatically increased the expression of TLR2 at both the messenger RNA and protein levels. In addition, TLR2 deficiency significantly inhibited chronic morphine-induced apoptosis in primary neurons. Activation of caspase-3 after morphine treatment is impaired in TLR2 deficient primary neurons. Moreover, morphine treatment failed to induce an increased level of phosphorylated glycogen synthase kinase 3 beta (GSK3β) in TLR2 deficient primary neurons, suggesting an involvement of GSK3β in morphine-mediated TLR2 signaling. These results thus demonstrate that opioids prime neurons to undergo apoptosis by inducing TLR2 expression. Our data suggest that inhibition of TLR2 is capable of preventing opioids-induced damage to neurons.

Introduction

Opioids have been widely applied in clinics as one of the most potent pain relievers for centuries. However, chronic use of opioids as clinical therapies produces benefits as well as severe side effects. Opioids, including morphine, cause apoptosis in various systems. We have previously reported that morphine promotes apoptosis both *in vitro* and *in vivo* [1–3]. In central nervous system (CNS), opioids-induced neuronal apoptosis [4]. Although opioid receptors play critical roles in the processes of opioids-induced effects, the antagonists of opioid receptors can only partially block the effects of opioids [5]. Thus, the specific cellular and molecular mechanisms underlying on opioids-induced apoptosis still need to be defined.

Toll-like receptors (TLRs) are well known as recognition of pathogens in the innate immune system aimed as defending the survival of the host. Thirteen TLRs have been identified [6]. TLRs and their functions have been established in immune cells. However, the functional role of TLRs in the CNS remains unclear. Growing evidence demonstrated that neurons express some TLRs, including TLR2, TLR4, and TLR9 [7]. Neuronal TLRs play pivotal roles in brain injuries and functional deficits [7,8]. TLR2 was identified as a key immune receptor in TLRs family with a large repertoire of ligands. Many classes of microorganisms, as well as the bacterial cell wall components peptidoglycan and lipoteichoic, have been found to activate TLR2. Activation of TLR2 signaling triggers activation of proapoptotic signals, and causes cell death in various systems [7,8]. Caspase activities increased significantly in TLR2 signaling activated cells [9,10].

Recent evidence suggests that there is cross-talk between TLR signaling and glycogen synthase kinase 3 (GSK3), a crucial regulator of many cellular functions including cell survival and apoptosis [9,11]. GSK3 is a serine/threonine kinase that refers to two isoforms – GSK3α and GSK3β [9,11]. It is considered that GSK3 promotes the mitochondrial intrinsic apoptotic signaling cascade induced by a diverse array of insults [9,11,12]. On mechanisms tightly regulating the activities of two isoforms of GSK3, the most well-defined mechanism is the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway. GSK3 activity is inhibited through PI3K/Akt signaling by phosphorylation of serine-9 in GSK3β or serine-21 in GSK3α [9,12].

In present study we found that TLR2 is required for morphine-induced neuronal cell death and apoptosis. Furthermore, Morphine failed to induce an increased level of phosphorylated GSK3β in TLR2 deficient primary neurons, suggesting an involvement of GSK3β in morphine-mediated TLR2 signaling.

* Corresponding authors. Fax: +1 423 439 6387.
E-mail addresses: docpengy@yahoo.com.cn (Y. Peng), yin@etsu.edu (D. Yin).
[1] These authors Contributed equaly to this work.

0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.11.074

Materials and methods

Reagents. Morphine sulfate was obtained from Sigma–Aldrich. Cell culture medium, horse serum, B27 supplement and reagents for neuron cell culture were purchased from Invitrogen Corporation. The quantitative PCR kit was purchased from Invitrogen Corporation. The polyclonal anti-cleaved caspase-3, caspase-3, p-Ser9-GSK3β, total-GSK3β, p-Akt, and GAPDH antibodies were purchased from Cell Signaling Technology. The monoclonal TLR2 antibody was obtained from Santa Cruz Biotechnology Inc.

Animals. Toll-like receptor 2 knockout (TLR2 KO) mice on a C57BL/6 background and wild type C57BL/6 (WT) mice were obtained from the Jackson Laboratory and were maintained in the Division of Laboratory Animal Resources at East Tennessee State University (ETSU), a facility accredited by the Association fro the Assessment and Accreditation of Laboratory Animal Care International (AAALAC). All aspects of the animal care and experimental protocols were approved by the ETSU Committee on Animal Care. Pregnancy was confirmed by the presence of vaginal plug and this was considered as gestational day 0 (E0).

Primary cortical neuron culture. The method used for preparing primary cortical neuron cultures followed the procedure described in our previous publication with a slight modification [13]. Briefly, pregnant mice on E16 were anesthetized with carbon dioxide and killed by cervical dislocation. Meninges-free cortices were dissected from the 16-day-old fetuses and triturated into homogeneous solution. The cells were plated with a density of 1×10^6/well on 6-well-plates precoated with 100 μg/ml poly-D-lysine. The cells were cultured with neurobasal medium supplemented with 5% horse serum, 2% B27, 1% glutamax and 50 μ/ml penicillin/streptomycin, and were kept in a tissue culture incubator at 37 °C in 5% CO_2. Seven-day-old cortical neurons were subjected to morphine treatment with different concentrations within 6 days.

Determination of cell death. Neuronal cell density was observed directly with a microscope during drug treatment. At the end point of each group, neurons in some wells were stained with propidium iodide (PI) solution as described previously [2] and were observed with a fluorescent microscope (Motic Company). To determine the change of cell death in wells, five areas in each well were randomly chosen for cell counting. Data were normalized from control-treated cells and shown as a percentage of the controls.

Detection of apoptosis by TUNEL assay. Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assay was detected as described previously [3]. Neurons treated with or without 15 μM morphine for 6 days underwent DNA fragmentation detection using an in situ apoptosis detection kit (Roche) according to the manufacturer's instructions. Cells were observed using a fluorescent microscope (Olympus).

Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR). The real-time PCR detection technique was performed as described in our previous publication [14]. Briefly, first-strand cDNA was synthesized from 1 μg of total RNA using a Reaction Ready™ first-strand cDNA synthesis kit (SuperArray Bioscience Corporation). After incubation at 70 °C for 3 min and cooling down to 37 °C for 10 min, RT cocktail was added to the annealing mixture and further incubated at 37 °C for 60 min. Two microliters of 1:2 diluted cDNA was subjected to real-time quantitative PCR using Bio-Rad iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad). PCR was performed in a 25 μl volume using SYBR GreenER qPCR Super Mix for iCycler (Invitrogen). All primers were purchased from SuperArray Bioscience Corporation. All PCR assays were performed in triplicate. The reaction conditions were: 50 °C for 2 min, 95 °C for 8 min 30 s, followed by 40 cycles of 95 °C for 15 s, 60 °C for 60 s, and 72 °C for 30 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified from all samples on each plate as housekeeping genes. The reaction mixtures without template cDNA were used as negative controls.

Western blot analysis. Western blotting was performed as described in our previous publication using several antibodies [9,12]. Briefly, experimental cells were harvested and lysed in RIPA Lysis Buffer. The lysates were separated by 10% SDS–PAGE then transferred to a nitrocellulose membrane (Bio-Rad). The membrane was then incubated at room temperature in blocking solution for 1 h, followed with the blocking solution containing first antibody overnight at 4 °C. Caspase-3, p-Ser9-GSK3β, total-GSK3β, p-Akt, or GAPDH was detected with antibody specific to p-Ser9-GSK3β, total-GSK3β, p-Akt, or GAPDH. After washing three times with TBS for 5 min, the blot was incubated with a second antibody. The blot was again washed three times with TBS before being exposed to the SuperSignal West Dura Extended Duration substrate (Pierce).

Immunohistochemistry. Primary neuron cells were fixed and immunostained as described in our previous publication, with slightly modification [13]. Briefly, for TLR2 immunostaining, fixed neurons were immunostained using anti-TLR2 Ab and Alexa Fluor 488 (Invitrogen). For cleaved Caspase-3 and NeuN double immunostaining, fixed neurons were immunostained using anti-NeuN and anti-cleaved caspase-3 antibodies, followed by Alexa Fluor 488 or Alexa Fluor 594 conjugated secondary antibodies. For p-Ser9-GSK3β, fixed neurons were immunostained using anti-p-Ser9-GSK3β Ab and Alexa Fluor 594 (Invitrogen). Confocal images were captured using a Leica TCS SP2 Laser Scanning Confocal Microscope. Fluorescent images were captured using a fluorescent microscope (Olympus).

Statistical analysis. All data were represented as means ± SEM. The data were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni tests to determine where differences among groups existed. Differences were considered statistically significant for values of $p < 0.05$.

Results

Increased TLR2 expression in wild type neurons following chronic morphine treatment

Although it is well established that several opioid receptor isoforms are expressed on neurons, the mechanisms by which opioids influence neuronal function remains to be elucidated [15]. To investigate the mechanisms, we treated primary mouse cortical neurons from wild type mice with morphine sulfate at 15 μM for different periods of time and examined the expression of TLR2 by quantitative real time RT-PCR and immunohistochemistry. TLR2 expression in mRNA level was significantly increased following 15 μM morphine treatment for 4 days (Fig. 1A). Morphine-induced TLR2 expression was also detected at the protein level (Fig. 1B). Our results demonstrated that chronic morphine treatment significantly increases TLR2 expression.

A deficiency of TLR2 is resistant to morphine-induced neuronal apoptosis

It has been reported that cortical neurons generated a significant reduction in cell density following 15 μM opioid treatment for 7 days [4]. To investigate whether TLR2 plays a role in opioids-induced neuronal cell death, we treated primary mouse cortical neurons from TLR2 knockout mice and wild type mice with morphine at different concentrations for 6 days and assessed cell death using PI staining. We found that chronic morphine treatment induced cell death in a dose-dependent manner in wild type neu-

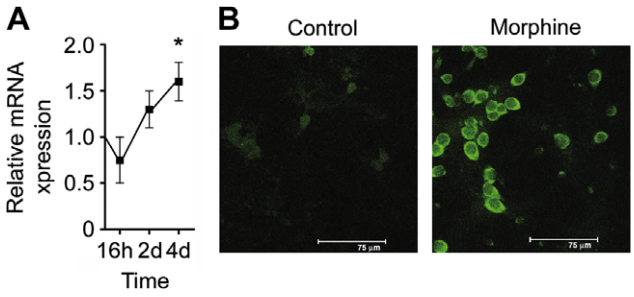

Fig. 1. Morphine induces the expression of TLR2 in wild type neurons. (A) Quantitative real time RT-PCR analysis of TLR2 expression showed a significant increase in wild type primary cortical neurons treated with morphine for 4 days. Neurons were treated with 15 μM morphine for 16 h, 2 d and 4 d, respectively. * $p < 0.05$ vs. untreated neurons. Results represent mean ± SEM of three independent experiments. (B) Immunostaining in wild type neurons showed a dramatic increase of TLR2-immunoreactive neurons after 15 μM morphine treatment for 6 days. Scale bars, 75 μm.

rons, but not in TLR2 deficient neurons (Fig. 2A). Dead cell rate showed no significant differences among 5, 15 μM morphine treated TLR2 deficient neurons and untreated neurons. We next evaluated the effect of TLR2 on morphine-induced primary neuronal apoptosis using a TUNEL technique. A significant number of cells in the wild type neurons after morphine treatment were undergoing apoptosis, whereas only a few apoptotic cells were detected in the TLR2 deficient neurons following morphine treatment (Fig. 2B). Therefore, primary neurons with a deficiency of TLR2 are resistant to morphine-induced neuronal apoptosis.

Morphine causes caspase-3 activation in wild type neurons but not in TLR2 deficient neurons

The cleaved caspase-3 acts as an active and lethal protease at the most distal stage of the apoptotic pathways, and is the major caspase involved in neuronal apoptosis [16]. Thus, we examined caspase-3 activation in TLR2 deficient and wild type primary neurons after morphine treatment. Morphine treatment at 15 μM for 6 days activated caspase-3 in wild type neurons to a greater increase compared to TLR2 deficient neurons (Fig. 3A). These results were verified by double-immunofluorescent staining of active caspase-3 and neurons (Fig. 3B). Morphine treatment at 15 μM for 6 days resulted in a dramatic increase of cleaved caspase-3 immunoreactive colocalization in wild type primary neurons, but not in TLR2 deficient neurons. Our results suggested a proapoptotic role for TLR2 in opioids-mediated neuronal apoptosis.

TLR2 deficiency in neurons blocks morphine-enhanced phosphorylation of GSK3β

We have previously reported that TLRs activate GSK3β signaling pathways [12]. To substantiate that opioids activate TLR2 signaling, we investigated the regulation of GSK3β in morphine-activated TLR2 signaling. As shown in Fig. 4A, GSK3β was phosphorylated on the inactivating residue serine-9 and phosphorylation significantly increased after 15 μM morphine treatment for 6 days in wild type neurons. A deficiency of TLR2 in primary neurons strongly suppressed chronic morphine-induced GSK3β serine-9 phosphorylation (Fig. 4A). The changes of GSK3β serine-9 phosphorylation were verified by immunohistochemistry. Morphine treatment resulted in a dramatic increase of GSK3β serine-9 phosphorylation on protein level in wild type primary cortical neurons, but not in TLR2 deficient primary cortical neurons (Fig. 4B). Since phosphorylation of serine-9 is inhibitory for GSK3β activity, these results suggested that morphine decreases GSK3β activity through a TLR2-dependent mechanism.

Akt is an important physiologic mediator of the PI3K pathway [2,17]. Activated Akt phosphorylates several downstream targets of the PI3K pathway including GSK3 [2,17]. Therefore, we determined if morphine induces Akt phosphorylation through a TLR2-dependent pathway. We found no alterations in the protein level of phosphorylated Akt in wild type neurons and TLR2 deficient neurons following morphine treatment (Fig. 4C), suggesting no role of Akt in morphine suppression of TLR2-mediated GSK3β activity.

Discussion

The data presented herein demonstrated for the first time, to our knowledge, a key role for neuronal TLR2 in the induction of morphine-mediated apoptosis. Our studies show that chronic morphine treatment regulates TLR2 signaling and results in neuronal apoptosis. It is widely accepted that TLR2 signaling mainly activates inflammatory responses, including proinflammatory cytokines [18]. Most studies evaluated the effects of TLR2 on neuronal cell death and apoptosis through immune cell mediated mechanisms [19]. We found in this study that deficiency of TLR2 activity in primary neurons blocked morphine-induced neuronal apoptosis. We identified here that TLR2 signaling is activated by opioids in neurons without the presence of immune cells. This provides a possible target for controlling of morphine caused neurotoxicity.

We have previously reported that TLRs activate phosphorylation of GSK3β [12]. GSK3 activities cause diverse regulation of

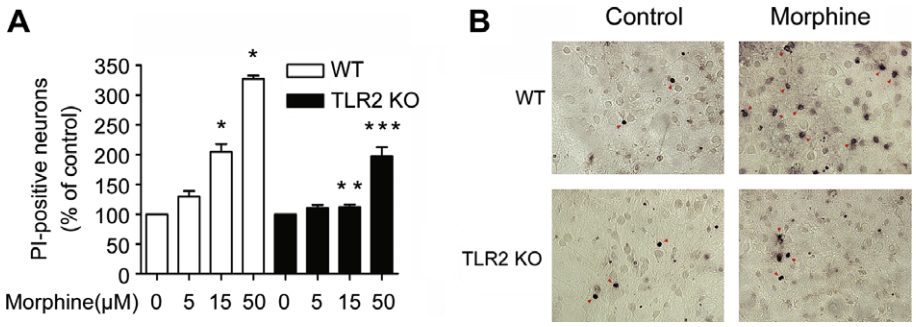

Fig. 2. A deficiency of TLR2 is resistant to morphine-induced neuronal apoptosis. (A) Bar graph showed the percentage of neuronal cell death in wild type (WT) and TLR2 knockout (TLR2 KO) neurons identified with PI staining. WT and TLR2 KO neurons were treated with 5, 15, or 50 μM morphine for 6 days, respectively. Results represent mean ± SEM of three independent experiments. * $p < 0.05$ vs. untreated WT neurons. ** $p < 0.05$ vs. WT neurons treated with 15 μM morphine. *** $p < 0.05$ vs. WT neurons treated with 50 μM morphine. (B) Representative light microscopic images showed TUNEL-positive neurons. WT and TLR2 KO neurons were treated with or without 15 μM morphine for 6 days. The dark brown color showed TUNEL-positive neuronal apoptosis (red arrow head). Magnification 200×. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

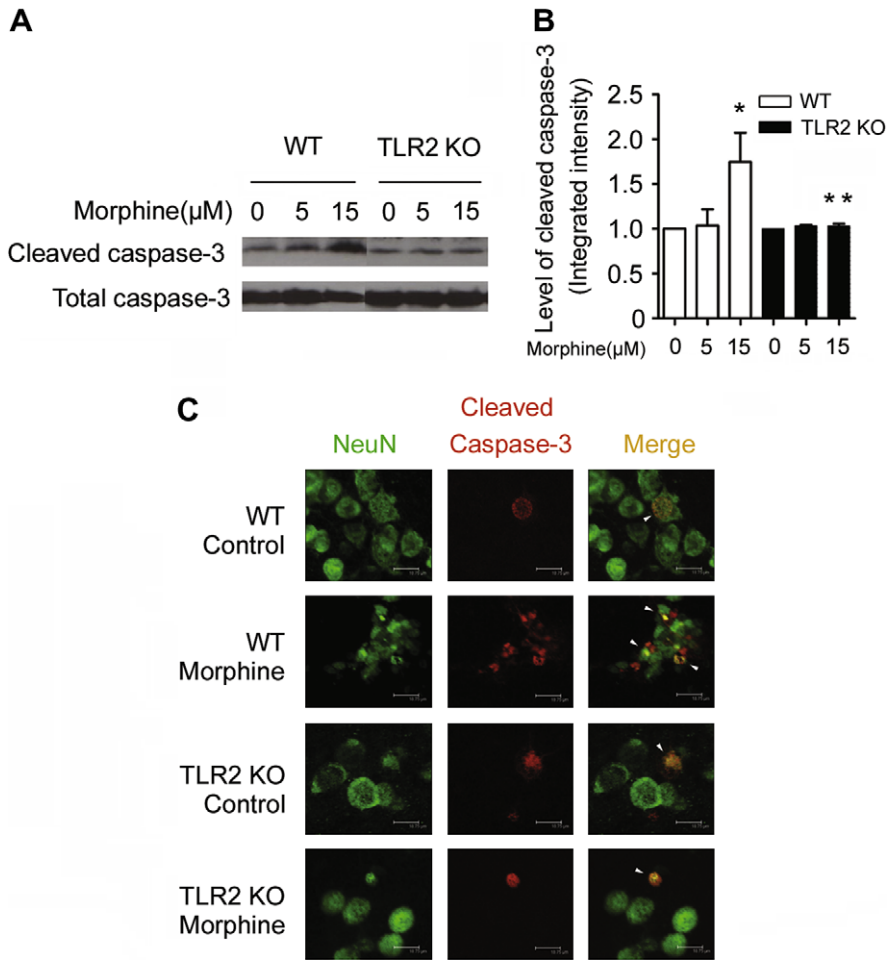

Fig. 3. Morphine causes caspase-3 activation in wild type neurons but not in TLR2 knockout neurons. (A) Total and active cleaved caspase-3 in protein levels were determined by Western blot. WT and TLR2 KO neurons were treated with morphine at 5 or 15 μM for 6 days. Representative results of the level of cleaved caspase-3 and total caspase-3 are shown at the left of each pane. Data are representative of three independent experiments. $^{*}p < 0.05$ vs. untreated WT neurons. $^{**}p < 0.05$ vs. WT neurons treated with 15 μM morphine. (B) WT and TLR2 KO neurons were treated with or without 15 μM morphine for 6 days and immunostained with antibodies to cleaved caspase-3 (red) and NeuN (neuronal marker) (green). Merged images displayed co-localizations of cleaved caspase-3 and NeuN (yellow) (white arrow head). Scale bars, 18.75 μm. Data are representative of three separate experiments.

TLR-mediated production of pro- and anti-inflammatory cytokines [17,18]. Selective agonists of TLRs, mediate phosphorylation of GSK3β on Ser9, and result in GSK3β inactivation. In agreement with these results, our data showed that morphine decreased GSK3β activity through a TLR2-dependent mechanism. Akt activates several downstream targets, such as GSK3β. Interestingly, we found no role of Akt in morphine suppression of TLR2-mediated GSK3β activity. It remains to be determined whether other kinases phosphorylate GSK3β. Furthermore, scaffolding proteins provide one possible mechanism on interactions between these kinases. Our previous results also reveal that a scaffolding protein β-arrestin 2 is involved in TLR2-mediated apoptosis [10].

In our study, we found that chronic morphine decreases GSK3β activity. These evidences lead us to propose that GSK3β activity protects from opioids-induced neuronal apoptosis. We reported previously that morphine modulates apoptosis through cell death receptor Fas, the other major member of the death receptor family [1]. Apoptosis signaling induced by Fas was found to be regulated by the anti-apoptotic effect of GSK3 [20]. Further investigations should be performed to understand the mechanisms on GSK3 activities associated with opioids-mediated apoptosis. We showed that morphine induces neuronal cell death and apoptosis in a dose-dependent manner. These data are consistent with the findings of Svensson and Hu [4,5]. Furthermore, we found that in the deficiency of TLR2, morphine induced effects on neuronal cell death and caspase-3 activity, were counteracted. Thus, a caspase-3-dependent pathway is involved in TLR2 signaling activated by chronic morphine effects. Evidences have already shown that activation of caspase-3 is a terminal event leading to neuronal apoptosis upon chronic exposure to morphine, and there may be changes in intracellular proapoptotic elements such as Bax, Fas, and anti-apoptotic elements such as Bcl-2 in response to chronic morphine effects [5]. The cleaved caspase-3 acts as an active and lethal protease at the most distal stage of the apoptotic pathways, and is the major caspase involved in neuronal apoptosis [16]. The extrinsic pathway, one of the two identified major caspase-3-activating pathways, involves cell death receptors [21]. In our study, TLR2 deficiency in neurons reversed the caspase-3 activity increasing effect induced by chronic morphine treatment. We identified that downstream of TLR2, caspase-3 as a crucial factor for morphine-induced neuronal apoptosis.

In summary, our studies reveal that TLR2 plays a critical role in opioids-induced neuronal apoptosis and an involvement of GSK3β. Further understanding the mechanisms mediated by TLRs may provide insights into potential therapeutic interventions opioid-related neurotoxicity.

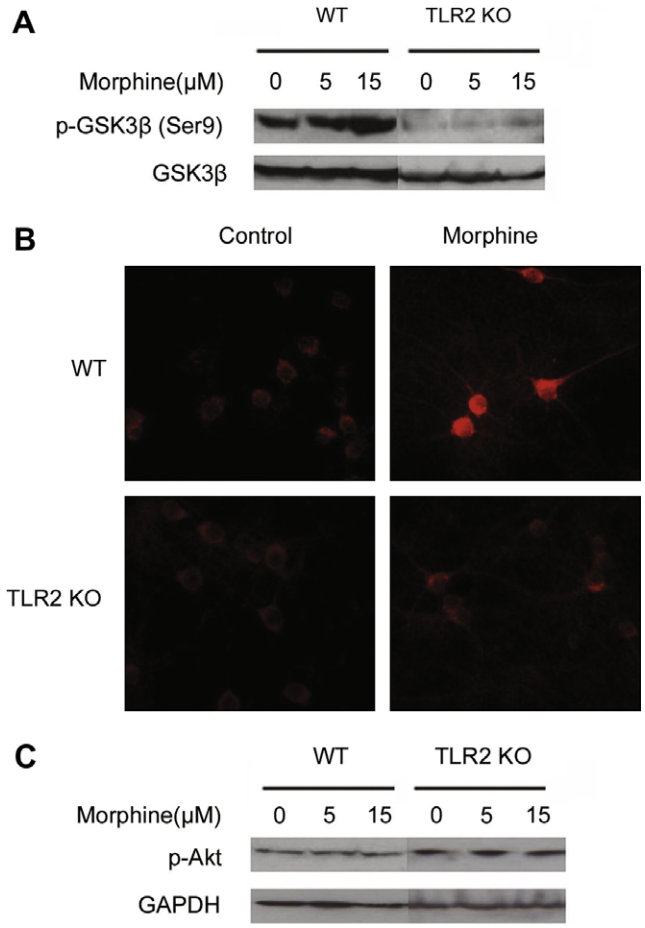

Fig. 4. TLR2 deficiency in neurons blocks morphine-enhanced GSK3β serine-9 phosphorylation. (A) Phosphorylated serine-9 GSK3β (p-Ser9-GSK3β) and total-GSK3β in protein levels were examined by Western blot. WT and TLR2 KO neurons were treated with 5 or 15 μM morphine for 6 days, respectively. Data are representative of three independent experiments. (B) WT and TLR2 KO neurons were treated with or without 15 μM morphine for 6 days and immunostained with p-Ser9-GSK3β antibody. (C) Phosphorylated Akt (p-Akt) was determined by Western blot. We treated WT and TLR2 KO primary neurons with or without 15 μM morphine for 6 days and examined for the level of phosphorylated Akt (p-Akt). Data are representative of three independent experiments.

Acknowledgments

This work was supported by National Institutes of Health (NIH) Grant DA020120 and East Tennessee State University Research Development Committee (ETSU RDC) 0048 to D. Yin. This work was also supported in part by ETSU RDC Grant 07-026M to G. Hanley.

References

[1] D. Yin, R.A. Mufson, R. Wang, Y. Shi, Fas-mediated cell death promoted by opioids, Nature 397 (1999) 218.
[2] D. Yin, M. Woodruff, Y. Zhang, S. Whaley, J. Miao, K. Ferslew, J. Zhao, C. Stuart, Morphine promotes Jurkat cell apoptosis through pro-apoptotic FADD/P53 and anti-apoptotic PI3K/Akt/NF-kappaB pathways, J. Neuroimmunol. 174 (2006) 101–107.
[3] D. Yin, D. Tuthill, R.A. Mufson, Y. Shi, Chronic restraint stress promotes lymphocyte apoptosis by modulating CD95 expression, J. Exp. Med. 191 (2000) 1423–1428.
[4] A.L. Svensson, N. Bucht, M. Hallberg, F. Nyberg, Reversal of opiate-induced apoptosis by human recombinant growth hormone in murine foetus primary hippocampal neuronal cell cultures, Proc. Natl. Acad. Sci. USA 105 (2008) 7304–7308.
[5] S. Hu, W.S. Sheng, J.R. Lokensgard, P.K. Peterson, Morphine induces apoptosis of human microglia and neurons, Neuropharmacology 42 (2002) 829–836.
[6] S.L. Doyle, L.A. O'Neill, Toll-like receptors: from the discovery of NFkappaB to new insights into transcriptional regulations in innate immunity, Biochem. Pharmacol. 72 (2006) 1102–1113.
[7] S.C. Tang, T.V. Arumugam, X. Xu, A. Cheng, M.R. Mughal, D.G. Jo, J.D. Lathia, D.A. Siler, S. Chigurupati, X. Ouyang, T. Magnus, S. Camandola, M.P. Mattson, Pivotal role for neuronal Toll-like receptors in ischemic brain injury and functional deficits, Proc. Natl. Acad. Sci. USA 104 (2007) 13798–13803.
[8] I. Sabroe, S.K. Dower, M.K. Whyte, The role of Toll-like receptors in the regulation of neutrophil migration, activation, and apoptosis, Clin. Infect. Dis. 41 (Suppl. 7) (2005) S421–S426.
[9] L. Chen, Y. Zhang, X. Sun, H. Li, G. LeSage, A. Javer, X. Zhang, X. Wei, Y. Jiang, D. Yin, Synthetic resveratrol aliphatic acid inhibits TLR2-mediated apoptosis and an involvement of Akt/GSK3beta pathway, Bioorg. Med. Chem. 17 (2009) 4378–4382.
[10] Y. Li, X. Sun, Y. Zhang, J. Huang, G. Hanley, K.E. Ferslew, Y. Peng, D. Yin, Morphine promotes apoptosis via TLR2, and this is negatively regulated by beta-arrestin 2, Biochem. Biophys. Res. Commun. 378 (2009) 857–861.
[11] M. Guha, N. Mackman, The phosphatidylinositol 3-kinase-Akt pathway limits lipopolysaccharide activation of signaling pathways and expression of inflammatory mediators in human monocytic cells, J. Biol. Chem. 277 (2002) 32124–32132.
[12] Y. Zhang, Y. Zhang, J. Miao, G. Hanley, C. Stuart, X. Sun, T. Chen, D. Yin, Chronic restraint stress promotes immune suppression through toll-like receptor 4-mediated phosphoinositide 3-kinase signaling, J. Neuroimmunol. 204 (2008) 13–19.
[13] X. Lv, L. Su, D. Yin, C. Sun, J. Zhao, S. Zhang, J. Miao, Knockdown of integrin beta4 in primary cultured mouse neurons blocks survival and induces apoptosis by elevating NADPH oxidase activity and reactive oxygen species level, Int. J. Biochem. Cell Biol. 40 (2008) 689–699.
[14] D. Yin, Y. Zhang, C. Stuart, J. Miao, Y. Zhang, C. Li, X. Zeng, G. Hanley, J. Moorman, Z. Yao, M. Woodruff, Chronic restraint stress modulates expression of genes in murine spleen, J. Neuroimmunol. 177 (2006) 11–17.
[15] C.L. Stucky, M.S. Gold, X. Zhang, Mechanisms of pain, Proc. Natl. Acad. Sci. USA 98 (2001) 11845–11846.
[16] F. Hua, J. Ma, T. Ha, Y. Xia, J. Kelley, D.L. Williams, R.L. Kao, I.W. Browder, J.B. Schweitzer, J.H. Kalbfleisch, C. Li, Activation of Toll-like receptor 4 signaling contributes to hippocampal neuronal death following global cerebral ischemia/reperfusion, J. Neuroimmunol. 190 (2007) 101–111.
[17] T. Fukao, S. Koyasu, PI3K and negative regulation of TLR signaling, Trends Immunol. 24 (2003) 358–363.
[18] S. Akira, K. Takeda, Toll-like receptor signalling, Nat. Rev. Immunol. 4 (2004) 499–511.
[19] P.A. Carpentier, D.S. Duncan, S.D. Miller, Glial toll-like receptor signaling in central nervous system infection and autoimmunity, Brain Behav. Immun. 22 (2008) 140–147.
[20] L. Song, T. Zhou, R.S. Jope, Lithium facilitates apoptotic signaling induced by activation of the Fas death domain-containing receptor, BMC Neurosci. 5 (2004) 20.
[21] A.G. Yakovlev, K. Ota, G. Wang, V. Movsesyan, W.L. Bao, K. Yoshihara, A.I. Faden, Differential expression of apoptotic protease-activating factor-1 and caspase-3 genes and susceptibility to apoptosis during brain development and after traumatic brain injury, J. Neurosci. 21 (2001) 7439–7446.