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Changes in opioid receptor proteins during mitochondrial impairment in differentiated SK-N-SH cells

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Abstract

Aging and neurodegenerative diseases are associated with oxidative damage that may contribute to changes in neurosensory processing, including pain. The effects of neuronal oxidation on the opioid receptor system are poorly understood. Earlier, we have reported that 3-nitropropionic acid (3-NPA)-induced oxidative stress and impairment of mitochondrial energy metabolism significantly reduced the function of *mu* but not *delta* opioid receptors [Neurosci Letter 404 (2006) 242-246]. In the present study, we studied the effects of 3-NPA-induced oxidative stress on protein levels of the *mu*, *delta* and *kappa* opioid receptors (MOR, DOR and KOR, respectively). The opioid-responsive differentiated SK-N-SH neuronal cells were used as an *in vitro* model. Cells were exposed to 0, 5, 10 and 20 mM of 3-NPA for 0, 1, 2, 12, and 24 hours. After the 3-NPA treatments, plasma membrane preparations were made and used for the Western blot assay. There was a significant reduction in the level of the MOR protein while levels of DOR and KOR proteins remained unaffected after exposure to 3-NPA. These findings demonstrate for the first time that there is a selective impairment of the MOR protein under conditions of mitochondrial oxidative damage at the neuronal level. The reduction in the level of the MOR protein may contribute to the impairment of MOR function under oxidative damage conditions shown in our previous study.

Keywords

opioid receptors; proteins; oxidative stress; 3-nitropropionic acid

The deleterious effects of reactive oxygen species are responsible for the deterioration of neuronal function in neurodegenerative pathologies and aging [10,17]. Since free-radical-induced oxidative stress leads to an impairment of neuronal function, it may have a detrimental effect on opioid receptors. Opioid receptors are essential in responses to pain and opioid analgesics. The functioning of the opioid receptor system under conditions associated with oxidative damage is poorly understood. The impaired sensitivity in other receptor systems, such as adrenergic and cholinergic, under oxidative stress conditions, was reported [15,16,

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18]. At present, we do not understand oxidative stress-induced changes in the opioid receptors system, i.e., *mu*, *delta*, and *kappa*, MOR, DOR, and KOR, respectively.

The 3-nitropropionic acid (3-NPA) is a mitochondrial toxin [22] and is used to induce oxidative stress in both *in vitro* and *in vivo* conditions [3,12]. Exposure to 3-NPA resulted in an increase of reactive oxygen species, induction of oxidative damage, and depletion of intracellular ATP [37,28]. We have established a neuronal model of oxidative stress induced by 3-NPA in differentiated, opioid-responsive SK-N-SH cells. In an earlier report, we have shown that an increase in reactive oxygen species in opioid-responsive neuronal cells selectively impaired the function of MOR [28]. These novel results demonstrated that the opioid receptor subtypes differ significantly in their sensitivity to mitochondrial damage. The present study continues to explore our previous findings on the effects of oxidative damage on three subtypes of opioid receptors (MOR, DOR and KOR) in opioid-responsive SK-N-SH cells.

In this project, we have used the same *in vitro* experimental model as in previous studies in which we investigated the function of opioid receptors [28]. The SK-N-SH cells differentiate to a neuronal phenotype [25] with an increased expression of MOR and DOR [39]. Differentiated SK-N-SH cells have been widely used as an experimental model to study opioid receptors [2,21,25,26,27,28].

The human neuroblastoma SK-N-SH cells (ATCC, Rockville, MD, USA) were grown in a monolayer at 37°C in a 5% CO₂ atmosphere in RPMI-1640 medium supplemented with streptomycin (100 mg/ml), penicillin (100 IU/ml), and 10% fetal bovine serum. The cells were plated at an initial density of 2×10^5 cells/ml in 100-mm culture plates. The medium was changed at 2-day intervals. The cells were differentiated with retinoic acid (10 µM) for 6 days [38].

On the day of the experiment, cells were exposed to medium containing 3-NPA (0, 5, 10, and 20 mM) for 0, 1, 2, 12, and 24 hrs [37]. After 3-NPA treatment, plasma membrane preparations were made as previously described [9]. The cells were washed in PBS buffer, scraped, centrifuged, resuspended in homogenization buffer (32 mM Tris-HCl, 16 mM sodium pyrophosphate, 1.6 mM EDTA, 0.8 mM EGTA, 0.64 mM NH₄ molybdate, 3.2 mM DTT) containing protease inhibitors (20 µg/ml leupeptin, 20 µg/ml aprotinin, 20 µg/ml soybean trypsin inhibitor and 40 µg/ml PMSF). The samples were quickly sonicated and the homogenate was centrifuged briefly to sediment unbroken cells and nuclei. The resultant pellet was dissolved in homogenization buffer containing proteinase inhibitors, Triton-X 100 (0.1%) and NP-40 (0.1%) by gently aspirating several times and incubating on ice for 1 hr. Protein concentration of the membranes was determined using the BCA method.

The MOR, DOR, and KOR proteins were measured by Western blots in plasma membrane preparations as previously described [33], with some modifications. Briefly, 10-40 µg of protein sample was mixed with SDS-sample buffer and incubated at 100°C for 5 min. SDS-PAGE was performed on 10% gels according to the procedures described previously [19] using a Bio-Rad electrophoresis unit. Protein was then transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) in transfer buffer at room temperature overnight using Bio-Rad electrophoretic transfer unit according to methods described by Towbin and colleagues [32]. The nitrocellulose membrane was washed, blocked with 5% nonfat milk blocking solution prepared in the TBS/T buffer and incubated overnight on orbital shaker with 1:10,000 primary antibody for MOR or DOR or KOR (rabbit polyclonal anti-MOR and anti-KOR antibodies, BioSource International, Inc. Camarillo, CA; and the primary rabbit polyclonal anti-DOR antibodies, Santa Cruz Biotechnology Inc., Santa Cruz, CA).

After incubation, the membrane was washed again and incubated for 30 minutes with 1:10,000 of the secondary anti-rabbit horse radish peroxidase labeled IgG antibodies (BioSource International, Inc., Camarillo, CA). After three washes with TBS/T, the blots were developed using the ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ). For stripping and reprobing, the membrane was incubated in stripping buffer at 45°C for 45 minutes, washed 4 times in the TBS/T buffer at room temperature and exposed to 5% nonfat milk blocking solution. To ensure accurate protein transfer, the blots were stripped and reprobed with primary antibody for β_1 subunit of Na^+/K^+ ATPase (1:2,500, Novus Biologicals, Inc. Littleton, CO). Densitometric analysis of the bands was done using the Scion image analysis software. The quantification of band intensity is represented as a percentage of the value of its corresponding control band in the same membrane preparation. Each set of experiments was repeated at least three times. Western immunoblots were quantified using scanning densitometry. The values depict normalized densitometric values as percent (%) control. Data are expressed as mean $\pm$ S.E.M. ($n = 4$). The statistical evaluation of data was performed by analysis of variance (ANOVA) followed by the Tukey post hoc test using GraphPad Prism Version 4.0 statistical software package. p value < 0.05 was considered as statistically significant.

In the initial experiment, the effect of a 2-hr treatment with 10 mM 3-NPA on the MOR, DOR and KOR proteins in plasma membrane of differentiated SK-N-SH cells was characterized. Western immunoanalysis of MOR proteins revealed a band at approximately 55 K_d , DOR proteins at 58 K_d and KOR proteins at 45 K_d (Fig. 1A). After treatment with 3-NPA, the level of MOR protein significantly decreased to $49 \pm 10\%$ control while the levels of DOR and KOR proteins did not change significantly (Fig. 1).

In a subsequent series of experiments, the effects of a 2-hr exposure to various doses of 3-NPA (0, 5, 10 and 20 mM) on protein levels of MOR, DOR and KOR were performed. After exposure to 10 mM and 20 mM of 3-NPA, the MOR protein significantly decreased to $43 \pm 7\%$ and $24 \pm 3\%$ of control, respectively (Fig. 2). After 3-NPA, levels of DOR and KOR proteins were not statistically different from the vehicle-treated cells.

To determine if the effect of 3-NPA depends on the duration of treatment, the cells were treated with 10 mM 3-NPA for 1, 2, 12 and 24 hrs and then the Western blot immunoassays were carried out. Exposure to 10 mM 3-NPA for 2, 12, and 24 hrs resulted in a significant reduction of MOR protein levels to $45 \pm 6\%$, $58 \pm 4\%$, and $36 \pm 3\%$ of control, respectively (Fig. 3). The DOR and KOR protein levels were insignificantly different from their controls.

In this study, we demonstrate for the first time a significant reduction in the membrane MOR protein level under conditions of impaired cell energy metabolism induced by 3-NPA and no change in DOR and KOR protein levels in opioid-responsive SK-N-SH neuronal cells. These findings imply that opioid receptor subtypes respond differently to the detrimental effect of cellular energy damage evoked by the interruption of mitochondrial function. In earlier studies conducted on the same cell model, we have shown that the function of MOR but not DOR was reduced under *in vitro* oxidative stress conditions induced by 3-NPA [28]. No literature reports, to our knowledge, have so far demonstrated the subtype-specific changes in the opioid receptor protein levels under conditions of mitochondrial oxidative damage. The novel findings from our current and previous studies show that opioid receptor subtypes respond differently to oxidative stress associated with mitochondrial damage and provide an initial insight into the mechanism(s) underlying the reduction in opioid receptor function.

Different amino acid sequences of MOR and DOR proteins [5] could contribute to the differences in sensitivity of these receptor subtypes under conditions of mitochondrial damage. The extracellular loops of opioid receptors consist of amino acid residues, such as cysteine and methionine that are sensitive to oxidation [20]. Therefore, differences in these amino acid

residues may contribute to the observed subtype-specific selectivity of oxidative damage on opioid receptors.

Opioid receptors are G protein-coupled receptors [12,34]. The opioid subtype-specific effects of oxidative stress may reflect differences in G proteins coupled to MOR and DOR. Studies with G_{α} -specific antibodies suggested that G_{i-2} proteins are coupled with DOR in NG 108-15 cells [23], while G_o proteins are coupled with MOR in SHSY-5Y cells [4]. G_o and G_{i-2} proteins may differ in their vulnerability to oxidative insult leading to damage in G_o but not G_{i-2} . Oxidation of G proteins can result in loss of anchoring of G proteins to opioid receptor in plasma membrane and lead to migration of opioid receptor to the cytosolic compartment. The differences in the sensitivity of G protein isoforms to oxidative damage could account for the observed differences in the effect of oxidative stress on MOR, DOR and KOR proteins.

The trafficking of MOR, DOR and KOR may also contribute to the observed differences. It has been reported that the internalization and trafficking of DOR and KOR are differentially regulated, even when activated by the same agonist [7]. It is not known if internalization of opioid receptors varies constitutively, i.e. in the absence of activation by an agonist. It seems likely that oxidative stress may influence the internalization and trafficking of MOR differently than DOR and KOR. In this case, more internalization of MOR under oxidative stress conditions could result in reduction in membrane bound MOR protein.

Opioid receptor proteins may become oxidized under conditions of mitochondrial damage. Studies have shown that free oxidative radicals cause oxidation of proteins in various regions of aged mammalian brain [24,31]. Our findings suggest that the MOR protein may be more vulnerable to oxidative damage than the DOR and KOR proteins. The effect of 3-NPA on MOR protein became significantly reduced after 2 hrs and remained as such at 24 hrs. To better understand this finding, future study needs to elucidate the role of the exposure time on the effects of 3-NPA on the MOR protein.

We investigated the membrane component of the cells because the opioid receptors are membrane bound G protein-coupled receptors and execute their activity through G proteins and adenylyl cyclase [12,34]. Specificity of antibodies used in the present study has not been established previously in membrane extracts of differentiated SK-N-SH cells but the bands obtained with these antibodies are consistent with those reported in the literature. The Western immunoanalysis of the MOR proteins revealed a band at approximately 55 K_d which is consistent with previous reports that describe molecular masses of 45 K_d to 75 K_d for the MOR [29,34]. For the DOR proteins, a band at approximately 58 K_d was obtained. This size is in agreement with the findings in the literature which describes molecular masses of 45 K_d to 65 K_d for the DOR [1,8,13,29,34]. The Western blot analysis of KOR showed a band at approximately 45 K_d which is consistent with other reports [6,29,30,34]. Western blots for β_1 Na^+ - K^+ ATPase expressed single or double bands migrating between 35-60 K_d , which was reported previously [35,36].

In the present study, opioid receptor proteins in the cytosolic component were not analyzed. It remains to be established if the opioid receptor proteins degrade and/or migrate from cell membrane to intracellular compartment under conditions induced by 3-NPA.

The reduction of the MOR protein level may explain our earlier findings on significant selective inhibition of MOR function under conditions of mitochondrial damage [28]. Thus, the decreased levels of the MOR protein may contribute to reduction of the MOR function observed during oxidative damage in SK-N-SH neuronal cells. No change in the protein level for DOR and KOR coincided with no change in the function of these two opioid receptor subtypes.

KOR in SK-N-SH cells has not been extensively studied, and to our knowledge, it has not been confirmed whether this cell line expresses KOR or if this receptor subtype is functional in these cells. Only one functional study reported that KOR agonists inhibited the amplitude of K^+ -induced $[Ca^{2+}]_i$ increase in SK-N-SH cells, but this effect was not blocked by KOR antagonist nor-binaltorphimine [14]. In the present study, in differentiated SK-N-SH cells, we have shown a band of approximately 45 K_d for KOR. Pilot studies on the function of KOR in differentiated SK-N-SH cells have found that spiradoline (selective KOR agonist) did not inhibit cAMP (unpublished data). This observation implies that if SK-N-SH cells express KOR, these receptors may not be functional, and hence, their contribution to cAMP inhibition might be insignificant. Another model needs to be employed to find out if/how oxidative damage alters the function of KOR.

Oxidative stress may not be the only factor contributing to the alterations in MOR protein levels after 3-NPA treatments observed in our study. Irrespective of oxidative damage, other consequences of reduced mitochondrial succinate dehydrogenase activity, e.g., reduced cellular ATP, could contribute to the changes in opioid receptor proteins. The results obtained to date from our previous and current studies provide directions for further research on the mechanism(s) underlying the subtype-specific effects of mitochondrial oxidative damage in opioid receptors.

In summary, the results of our study show for the first time that 3-NPA-induced oxidative mitochondrial damage is selectively detrimental to MOR but not DOR and KOR proteins in membranes from opioid-responsive SK-N-SH neuronal cells. The findings imply that opioid receptor subtypes differ significantly in their sensitivity to the consequences of mitochondrial impairment. Additional functional studies are needed to explore the effects of oxidative damage on opioid agonist-receptor interactions. As protein thiols that are present in opioid receptors [20] can be easily oxidized and such a modification is increasingly implicated in signal transduction, future studies should focus on the opioid receptor's protein thiol oxidative modification and on proteomic analysis of oxidatively modified opioid receptor proteins. The current and future research findings will help to better understand changes in the opioid receptor system under conditions associated with neuronal oxidative damage.

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Figure 1.

Western immunoassay of MOR, DOR and KOR proteins from membranes of differentiated SK-N-SH cells after treatment with 3-NPA (10 mM for 2 hrs). The wells were loaded with 30 μ g of proteins. Blots were stripped and reprobed with anti- β_1 Na⁺-K⁺ ATPase. (A) immunoblots of MOR, DOR, KOR and β_1 Na⁺-K⁺ ATPase. (B) Densitometric images of immunoblots. Columns depict normalized density values. * $p < 0.05$ vs. control (No NPA).

Figure 2.

Dose-response effects of 3-NPA on MOR, DOR and KOR proteins from membranes of differentiated SK-N-SH neuronal cells. The cells were incubated with 0, 5, 10 and 20 mM of 3-NPA for 2 hrs. (A) Western immunoblots of MOR, DOR, KOR and β_1 $\text{Na}^+ - \text{K}^+$ ATPase. All the wells were loaded with 30 μg of proteins. Blots were stripped and reprobed with anti- β_1 $\text{Na}^+ - \text{K}^+$ ATPase. (B) Densitometric images of immunoblots. Columns depict normalized density values. * $p < 0.05$ vs. control (0).

Figure 3.

Effects of the duration of treatment with 3-NPA on MOR, DOR and KOR proteins from membranes of differentiated SK-N-SH neuronal cells. The cells were incubated with 10 mM of 3-NPA for 0, 1, 2, 12 and 24 hrs. (A) Western immunoblots of MOR, DOR, KOR and β_1 Na^+/K^+ ATPase. All the wells were loaded with 30 μg of proteins. Blots were stripped and reprobed with anti- β_1 Na^+/K^+ ATPase. (B) Densitometric images of Western blots. Columns depict normalized density values. * $p < 0.05$ vs. control (0).