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Oxidative damage and sensitivity to nociceptive stimulus and opioids in aging rats

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Abstract

Oxidative stress contributes to aging and may cause alterations in pain and analgesia. Knowledge about effects of oxidative stress on the opioid system is very limited. This project was designed to determine the relationship between age-related oxidative damage and opioid antinociception. Three age groups of male Fischer 344 rats were tested for pain sensitivity and responses to morphine and fentanyl using the hot plate method. Oxidative stress markers in various brain regions were measured. With advancing age, nociceptive threshold and antinociceptive effects of opioids decreased significantly. There was a significant negative correlation between morphine antinociception and protein oxidation in cortex, striatum, and midbrain ($r^2 = 0.73, 0.87, \text{ and } 0.77$, respectively), and lipid peroxidation in cerebral cortex, hippocampus, and striatum ($r^2 = 0.73, 0.61 \text{ and } 0.71$, respectively). Similar correlation was observed between oxidative stress markers and fentanyl antinociception. These findings demonstrate that the age-related increase in oxidative damage in brain is associated with a significant decrease in the antinociceptive effects of opioids.

Keywords

age; opioids; pain; antinociception; oxidative stress markers

1. Introduction

Prevalence of pain is much higher in older than younger individuals. In the elderly (65 + years), the incidence of pain may be as high as 70% (Gloth, 1996). Opioid analgesics remain the mainstay of management of moderate to severe pain. Responses to the pain-relieving effect of opioids often show large variability in populations that differ in age. A few studies have shown that, in comparison to younger individuals, older patients require less morphine to achieve

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equivalent levels of analgesia (MacIntyre and Jarvis, 1996, Vigano et al., 1998). Furthermore, it was found that pain sensitivity is increased in elderly individuals (Moore et al. 1990).

A number of animal studies have reported age-related differences in pain sensitivity and antinociceptive response to opioids. There was a decline in morphine-induced antinociception in aged mice and rats (Chan and Lai, 1982, Hoskins et al., 1986, Kavaliers et al., 1983, Wallace et al, 1980). Other studies reported reduction and/or increase in morphine antinociception with aging (Saunders et al., 1974, Spratto and Dorio, 1978). The literature reports on the effects of aging on pain and opioids remain controversial.

Oxidative stress has been implicated in aging and neurodegenerative diseases (Floyd, 1999, Fukagawa, 1999; Kasapoglu and Ozben, 2001, Nohl, 1993). The aging process is associated with cellular damage caused by reactive oxygen species. There was a significant increase in protein oxidation in aged mice brain regions such as the cortex, hippocampus, striatum, and midbrain (Dubey et al., 1996, Forster et al., 2000, Rebrin et al. 2007), but a minimal or no change in the brainstem (Rebrin et al. 2007). The cortex, striatum, hippocampus, and midbrain express opioid receptors (Delfs, 1994, Mansour et al, 1988, Pasternak, 1993, Zastawny et al., 1994) and contribute to pain processing (Basbaum and Jessell, 2000), therefore age-related oxidative damage in these regions may affect pain sensitivity and opioid analgesia. Recent studies suggested a mediatory role of reactive oxygen species in pain associated with peripheral nerve injury (Crisp et. al, 2006, Hacimuftuoglu et al., 2006).

Previously, we have demonstrated that oxidative stress caused significant decrease in the function and protein level of opioid receptors in opioid-responsive SK-N-SH neuronal cells (Raut et al., 2006; Raut et al, 2007). The present study was designed to characterize the effects of oxidative stress damage that occurs during aging on changes in nociceptive threshold and responses to opioids, and the correlation between oxidative stress markers and opioid antinociception.

2. Methods

2.1. Animals

The experiments were performed in adherence with the guidelines of the Committee for Research and Ethical Issues of IASP. The protocol was approved by Institutional Animal Care and Use Committee (IACUC) of the University of North Texas Health Science Center. Male Fisher 344 rats of three different age groups, i.e. 3-6 months (young), 9-12 months (adult) and 21-24 months (old) were studied. The rats were housed under standard conditions within AAALAC accredited conditions (temperature $22 \pm 2^{\circ}\text{C}$ and light illuminated daily 0700-2000 hrs). Rats were provided tap water and laboratory chow *ad libitum*. During 10-day acclimatization, rats were handled daily.

2.2. Hot plate testing

Three age groups, 40 rats per group, were studied. Each age group was divided into four subgroups. Each subgroup received either vehicle (control) or one of three different doses of an opioid analgesic (morphine or fentanyl). First, the experiments were performed with morphine. After a one-week wash out period, similar experiments were performed with fentanyl. It was reported that repeated use of opioids did not lead to tolerance if the exposure was separated by 5-7 days (Cook et al., 2000, Walker et al., 1999). The rats in the control groups remained as controls in the cross over. The rats' responses to a thermal nociceptive stimulus at $56 \pm 0.1^{\circ}\text{C}$ were determined using the hot plate method. Hot plate testing was done at baseline and then 30, 45 and 60 minutes after morphine and 15, 30 and 45 minutes after fentanyl administration. During testing, the time lapse (in seconds) until front or hind paw lick

was recorded as hot plate latency (HPL). A cut off time of 30 seconds was used to avoid thermal trauma to the paws. To avoid diurnal variations in animal responses to a painful stimulus, all hot plate tests were performed between 0800 and 1100 hours. Experimental sessions were carried out in the animal housing room. Antinociceptive effect was calculated as % Maximum Possible Effect (%MPE) using the following formula: % MPE = [(Observed HPL) – (Baseline HPL)] / (30-Baseline HPL) × 100.

2.3. Drug administration

Morphine and fentanyl (Sigma Chemicals, St. Louis, MO) were administered subcutaneously. Selection of opioid doses was based on our pilot study and literature reports. Morphine was administered at doses of 5, 10, 15 mg/kg subcutaneously (Craft et al., 1995, Hoskins et al., 1986, Kramer and Bodnar, 1986). Fentanyl was injected at the doses of 25, 37.5, 50 µg/kg (Redwine and Trujillo, 2003, Rivat et al., 2002). During the study, each opioid-treated rat received a single injection of morphine and one week later, a single injection of fentanyl.

2.4. Dissection of brain regions

Six rats from each experimental group were randomly selected and sacrificed. The whole brain was quickly excised, placed on an ice cold Petri dish, and midbrain, striatum, hippocampus and cerebral cortex were dissected. The brain tissue was stored in antioxidant buffer (50 mM phosphate buffer (pH 7.4), 1 mM butylated hydroxytoluene (BHT), 100 µM diethylene triamine pentaacetic acid (DTPA) at –80°C.

2.5. Lipid peroxidation assay

Lipid peroxidation products were measured as thiobarbituric acid-reactive substances (TBARS) (Ohkawa et al., 1979). Thiobarbituric acid was added to the samples and the products were measured by fluorometry at excitation 525 nm and emission 550 nm. Concentrations of lipid peroxidation products were calculated by using standard solutions of thiobarbituric acid.

2.6. Protein oxidation assay

Protein carbonyl content, as a marker of protein oxidation, was measured as described previously (Levine et al., 1990). The differences in absorbance between samples were determined and the amount of carbonyl contents was calculated. Data are expressed as nmoles of carbonyls per mg of soluble extracted protein.

2.7. Statistical analysis

Data are expressed as mean ± S.E.M. Statistical comparisons were made between three different age groups of rats. 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. Correlation was studied with linear regression analysis and Pearson's coefficient (r) was calculated. *p* values <0.05 were considered as statistically significant.

3. Results

3.1. Effect of aging on thermal nociceptive threshold

The group differences in thermal nociceptive threshold are shown in Figure 1. The hot plate latency values in adult and old rats were significantly lower as compared to the young rats (young vs. adult *p* <0.05, young vs. old *p* < 0.001). The basal nociceptive threshold was not significantly correlated with body weight in young, adult and old rats. The average body weight were 326.7 ± 7.9 g in young rats, 384.1 ± 9.1 g in adult rats, and 391.8 ± 7.1 g in old rats.

3.2. Effect of aging on morphine-induced antinociception

There was a gradual increase in morphine-induced antinociception between 30 and 60 minutes (Figure 2). After 5 mg/kg of morphine, old rats showed significantly lower antinociception than young rats at 45 and 60 minutes (Figure 2A). After 10 mg/kg, the old rats showed significantly lower antinociception than young rats (Figure 2B). No significant group differences in morphine-induced antinociception were observed between young and adult rats at doses of 5 and 10 mg/kg. As shown on Figure 2C, the 15 mg/kg dose of morphine caused significantly lower antinociception in adult and old rats as compared to the young rats.

3.3. Effect of aging on fentanyl-induced antinociception

Maximum antinociceptive effects of fentanyl were observed at 30 minutes after administration. After 25 µg/kg of fentanyl, old rats showed significantly lower antinociception than young rats at 15, 30, and 45 minutes (Figure 3A). After a 37.5 µg/kg, old rats had significantly lower antinociception as compared to young rats (Figure 3B). No significant group differences in fentanyl-induced antinociception were observed between young and adult rats at doses of 25 and 37.5 µg/kg.

At 15 and 30 minutes, 50 µg/kg of fentanyl caused significantly lower antinociception in adult and old rats as compared to young rats (Figure 3C). At 45 minutes, the difference between antinociception in adult and young rats was insignificant.

3.4. Protein oxidation in brain regions

The levels of protein oxidation marker showed a gradual increase with aging in the cerebral cortex, hippocampus, striatum, and midbrain (Figure 4). In the cerebral cortex, old rats showed significantly higher carbonyl content as compared to young rats (Figure 4A). Similarly, protein carbonyl content in the hippocampus in old rats was significantly higher than in young rats (Figure 4B). Striatum showed the highest (approximately 3-fold) difference in protein carbonyl levels between young and old rats (Figure 4C). The adult and old rats had significantly higher levels of protein carbonyl content in striatum than young rats. In midbrain, significantly higher levels of protein carbonyls were measured in adult and old rats as compared to young rats (Figure 4D).

3.5. Lipid peroxidation in brain regions

TBARS content, a marker of lipid peroxidation, increased progressively with aging in the cerebral cortex, hippocampus, striatum and midbrain (Figure 5). In the cerebral cortex, old rats showed significantly higher levels of TBARS as compared to young rats (Figure 5A). Similarly, TBARS content in the hippocampus in old rats was significantly higher than in young rats (Figure 5B). Striatum had significantly higher levels of TBARS in adult and old rats as compared to young rats (Figure 5C). No significant group differences were observed in TBARS in the midbrain (Figure 5D).

3.6. Protein oxidation and opioid antinociception

There was a significant negative correlation between the antinociceptive effect of morphine (15 mg/kg at 60 minutes) and protein oxidation in the cortex, striatum, and midbrain ($r^2 = 0.73$, 0.87 , and 0.77 , respectively) but not in the hippocampus (Figure 6).

A significant negative correlation was observed between fentanyl-induced antinociception at 30 minutes after 50 µg/kg, and protein oxidation in the cortex, striatum and midbrain ($r^2 = 0.39$, 0.55 and 0.30 , respectively) but not in the hippocampus (Figure 7). Similar correlations were observed across other fentanyl doses and time points (data not shown).

3.7. Lipid peroxidation and opioid antinociception

Morphine-induced antinociception (15 mg/kg at 60 minutes) was significantly negatively correlated with lipid peroxidation in the cerebral cortex, hippocampus, and striatum ($r^2 = 0.73$, 0.61 and 0.71 , respectively) (Figure 8). In the midbrain, the negative correlation between lipid peroxidation and morphine antinociception was not significant. Similar correlation coefficients were found across other doses and time points for morphine.

There was a significant negative correlation between fentanyl-induced antinociception (50 $\mu\text{g/kg}$ at 30 minutes) and lipid peroxidation in the cerebral cortex, hippocampus and striatum ($r^2 = 0.27$, 0.39 and 0.26 , respectively) (Figure 9). In the midbrain, no significant correlation was observed between lipid peroxidation and fentanyl-induced antinociception. Correlation coefficients were similar across other doses and time points of fentanyl (data not shown).

4. Discussion

Many studies have reported that age changes responses to opioids, but have not analyzed these findings in relation to age-associated oxidative damage. The present project studied the effects of aging on responses to pain and opioids and on correlation between oxidative damage and opioid-induced antinociception. A significant negative correlation between responses to opioids (morphine and fentanyl) and oxidative stress markers in the brain was observed during aging. These findings imply a critical role of brain oxidative damage in the effects of opioids on pain.

Our present study shows a significant age-dependent decrease in pain threshold and antinociceptive responses to morphine and fentanyl in male Fisher 344 rats. A similar increase in sensitivity to pain and reduction in morphine analgesia was reported in 3-6 and 24-27 month-old male ICR mice (Hoskins et al., 1986), Charles River rats at 2, 5 and 10 months of age (Chan and Lai, 1982), and in 2-24 months old C57BL/6J mice (Webster, 1976). However, Smith and French (2002) have reported that there was no significant difference in baseline nociceptive sensitivity between young (3 months) and old (21 months) Fischer male rats when the warm water tail-withdrawal test was used.

Different methods of pain assessment measure nociception at different levels of the central nervous system. The tail-withdrawal test reflects spinal responses while the hot plate method determines supraspinal responses to painful stimulus (Dennis et al., 1980). Aging may cause a more profound decline in opioid system at supraspinal level than at spinal level, and therefore, account for the reported differences in pain parameters. Moreover, it was observed that morphine antinociception as measured by the vocalization test was higher in older (9-10 months) than younger (2-3 months) Sprague-Dawley rats (Saunders et al., 1974). Another study reported no differences in response to tactile-evoked stimulation between groups of 2-3 and 24-26 months old Fisher 344 FBNF1 rats (Crisp et al, 2006). Thus, the discrepancies among research reports may be attributed to differences in animal strain and/or in the methods used to measure pain.

The decreased nociceptive threshold and opioid antinociception observed in the present study may be due to multiple factors. Since aging rats gain body weight, higher body weight could lead to increased pressure between paw surface and the heated plate, and result in shortened paw withdrawal latency. Analysis of our data showed that there was no correlation between body weight and hot plate latency in any of the three age groups of rats.

In the light of the theory of enhanced oxidative stress during the aging process (Fukagawa, 1999, Nohl, 1993), the present study investigated possible association between age-related oxidative damage to the brain and opioid-induced antinociception. The brain regions studied in this project - cerebral cortex, striatum, midbrain, and hippocampus - are involved in pain

processing (Basbaum and Jessell, 2000), express opioid receptors (Pasternak, 1993, Zastawny et al., 1994), and, moreover, oxidative damage to proteins and lipids in these brain regions increase markedly with aging (Dubey et al., 1996, Forster et al., 2000, Liu et al., 2002, Rebrin et al. 2007). Recently, it was reported that reactive oxygen species play a mediatory role in spinal injury-induced pain (Crisp et al., 2006, Hacimuftuoglu et al., 2006).

In the present study, we found a significant increase in protein oxidation and lipid peroxidation in the cerebral cortex, hippocampus, striatum, and midbrain in old Fisher 344 rats. Increased protein oxidation in the brain was reported in old C57BL/6 mice (Dubey et al., 1996). Whole brain homogenates of old C57BL/6 mice (Forster et al., 2000) and SAMP8 mice (Farr et al., 2003) showed significantly higher protein carbonyl content as compared to young mice. Adult and old CD1 Swiss mice (Navarro et al., 2002) and Wistar rats (Navarro and Boveris, 2004) were shown to have higher levels of markers of lipid peroxidation in brain homogenates than their young controls. Thus, during aging there is a significant increase in oxidative damage mainly in the forebrain regions. To our knowledge, no information is available on oxidative stress damage that occurs during aging in the spinal cord.

Fentanyl and morphine antinociception showed a significant negative correlation with protein oxidation in the cerebral cortex, striatum, and midbrain. In the hippocampus, there was no correlation between protein oxidation and opioid antinociception. This may be due to lower opioid receptors density in hippocampus as compared to the other brain regions. In a pilot Western immunoassay, we could not achieve quantifiable bands for opioid receptor protein in the hippocampus (data not shown), suggesting low expression of opioid receptors in hippocampus. Protein oxidation in the hippocampus may have less impact on the decline in opioid antinociception. Moreover, a significant negative correlation between opioid antinociception and lipid peroxidation was found in the cerebral cortex, striatum, and hippocampus but not in the midbrain. Lipid peroxidation of the neuronal cell membranes leads to neuronal damage (Tatsumi and Fliss, 1994). Thus, neuronal damage in these brain regions may impair pain processing.

Oxidation of opioid receptor proteins can result in decreased opioid receptor function. One study reported significantly reduced binding density of mu opioid receptors (MOR) in the striatum in male Charles River rats treated with 3-nitropropionic acid, a mitochondrial toxin known to induce oxidative stress (Page et al., 2000). Our previous in vitro study performed on opioid-responsive SK-N-SH cells showed that oxidative stress significantly decreased the function of MOR but not delta opioid receptor (DOR) (Raut et al., 2006). Furthermore, in the same experimental cell model, a significant decrease in MOR protein level was observed (Raut et al., 2007). It is very likely that the effects of fentanyl and morphine observed in these animal experiments were mediated by the MOR.

Although the data show a correlation between oxidative stress markers and opioid antinociception, a number of factors could influence changes in opioid sensitivity observed in aging. Changes in opioid kinetics or dynamics, receptor density, testosterone level, or opioid receptor transduction mechanism could account for decreased opioid sensitivity in old rats. Additional experiments including spinal cord and brainstem should characterize the mechanism(s) underlying the differential responses to opioids observed during chronological aging. Subsequent animal or human studies should further elucidate the role of oxidative damage in sensitivity to opioids throughout the lifespan.

In conclusion, our findings show an age-related decrease in nociceptive threshold and opioid-induced antinociception. The reduction in opioid antinociception is significantly correlated with increased oxidative damage that develops in the brain during aging. These novel in vivo results together with our earlier in vitro findings strongly suggest that oxidative damage causes

changes in opioid receptors and eventually, results in decreased sensitivity to opioids. Impairments of the opioid system evoked by oxidative stress need to be further investigated to help us better understand age-related variability in pain and analgesia.

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Figure 1.

Effect of aging on baseline hot plate latency. The nociceptive threshold was measured in young (3-6 months), adult (9-12 months) and old (21-24 months) rats using the hot plate method at $56 \pm 0.1^\circ \text{C}$. $N = 40$ in each group. * $p < 0.05$, compared to young rats.

Figure 2.

Effect of aging on morphine-induced antinociception. The rats were administered (A) 5 mg/kg, (B) 10 mg/kg and (C) 15 mg/kg of morphine and responses to thermal nociceptive stimulus were measured 30, 45 and 60 minutes after morphine administration using the hot-plate method. $N = 10$ in each group. * $p < 0.05$, compared to young rats.

Figure 3.

Effect of aging on fentanyl-induced antinociception. Three different age groups of rats were administered (A) 25 $\mu\text{g/kg}$, (B) 37.5 $\mu\text{g/kg}$ and (C) 50 $\mu\text{g/kg}$ of fentanyl and antinociception was measured 15, 30 and 45 minutes after fentanyl administration using the hot-plate method. $N = 10$ in each age group. * $p < 0.05$, compared to young rats.

Figure 4.

Protein carbonyl content in (A) cerebral cortex, (B) hippocampus, (C) striatum, and (D) midbrain in young, adult, and old rats. The data are from 6 determinations on tissue homogenates from 6 individual rats. * $p < 0.05$, compared to young rats.

Figure 5.

Lipid peroxidation expressed as TBARS content in (A) cerebral cortex, (B) hippocampus, (C) striatum, and (D) midbrain rats of different age groups. The data are from 6 determinations on tissue homogenates from 6 individual rats. * $p < 0.05$ compared to young age group.

Figure 6.

Correlation between morphine-induced antinociception (expressed as % maximum possible effect, %MPE) and protein carbonyls in (A) cerebral cortex, (B) hippocampus, (C) striatum, and (D) midbrain. Linear regression relationship between protein carbonyls and morphine antinociception (15 mg/kg at 60 minutes) was analyzed and expressed as Pearson's coefficient. $N = 18$ (6 in each age group).

Figure 7.

Correlation between fentanyl-induced antinociception (expressed as % MPE) and protein carbonyls in (A) cerebral cortex, (B) hippocampus, (C) striatum, and (D) midbrain. Linear regression relationship between protein carbonyls and fentanyl antinociception (50 $\mu\text{g/kg}$ at 30 minutes) was analyzed and expressed as Pearson's coefficient. $N = 18$ (6 in each age group).

Figure 8.

Correlation between morphine-induced antinociception (expressed as %MPE) and lipid peroxidation (expressed as TBARS content) in (A) cerebral cortex, (B) hippocampus, (C) striatum, and (D) midbrain. Linear regression relationship between TBARS content and morphine antinociception (15 mg/kg at 60 minutes) was analyzed and expressed as Pearson's coefficient. $N = 18$ (6 in each age group).

Figure 9.

Correlation between fentanyl-induced antinociception (expressed as %MPE) and lipid peroxidation (expressed as TBARS content) in (A) cerebral cortex, (B) hippocampus, (C) striatum, and (D) midbrain. Linear regression relationship between TBARS content and fentanyl antinociception (50 $\mu\text{g/kg}$ at 30 minutes) was analyzed and expressed as Pearson's coefficient. $N = 18$ (6 in each age group).