Comparative Studies on Morphine- and Stress-Induced Analgesia and the Development of Tolerance to the Effects: Implication of Protein Synthesis Mechanism in the Process

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Abstract—Comparative studies were made on morphine- and stress-induced analgesia (SIA) and also on the development of tolerance to the effects. Cycloheximide (CYH), a potent protein synthesis inhibitor, did not affect the analgesic effect of morphine, but effectively suppressed the development of tolerance. CYH, however, potentiated both foot shock (FS) and immobilized-water immersion (IW) SIAs and inhibited the development of tolerance to FS-SIA. Incorporation of 3H-leucine into the TCA-insoluble fraction of mouse brain regions was inhibited by morphine, and the inhibition was reversed by the pretreatment with CYH. The inhibitory effect of morphine was lost in morphine tolerant animals. At the peak of SIA, incorporation of 3H-leucine was not changed in FS-SIA, but significantly inhibited in IW-SIA, and these effects were not modified by the pretreatment with CYH. The reduced incorporation of 3H-leucine in IW-SIA tolerant animals was partially reversed by CYH. Thus, the protein synthesis mechanism is greatly influenced by morphine or stresses, but the direct evidence for the implication of the mechanism in the process of producing analgesia and tolerance formation could not be demonstrated. However, differences in the underlying mechanisms were apparent between morphine and SIAs and also between FS- and IW-SIA.

The first suggestion by Cox et al. (1) that actinomycin D inhibits the development of analgesic tolerance to morphine without affecting its analgesic effect was followed by the reports on the possible involvement of the protein synthesis mechanism in the process (2, 3). Actually, various inhibitors of protein and nucleic acid synthesis modify the development and disappearance of tolerance to morphine (4, 5).

On the other hand, it has been well recognized that various stressful procedures induce an analgesic effect in experimental animals. In recent papers (6–9), it has been suggested that different systems, opioid and non-opioid forms, are involved in the mechanisms of the production of stress-induced analgesia (SIA).

In the present study, we compared the effect of cycloheximide on the production of morphine- and stress-induced analgesia and also on the development of tolerance to the effect. The inhibition of protein synthesis was monitored by determining the incorporation of radioactive leucine into the TCA-insoluble fraction of mouse brain regions in order to investigate the implication of the protein synthesis mechanism in the production of the analgesic effect and the development of tolerance to the effect.

Materials and Methods

Animals: Male mice of the dd-strain weighing 18 to 20 g were purchased and housed in a group of 10 animals in a plastic cage at an ambient temperature of 22±1°C. They were given normal laboratory diet and tap water ad libitum, and after reaching 23 to 26 g, they were used for the experiments.

Foot shock (FS) and immobilized-water
immersion (IW) stress: Details of these procedures have been described elsewhere (9, 10).

Drugs: Morphine-HCl (Mor, Takeda Pharm. Co.) and cycloheximide (CYH, Nakarai Chem. Co.) were dissolved in saline and administered intraperitoneally in a volume of 0.1 ml/10 g of body weight. CYH (10 mg/kg) was injected 2 hr prior to the administration of morphine or to the exposure to the stress.

Assessment of analgesic effect: The analgesic effect was assessed by the modified Haffner's method (11), every 15 min after the injection of morphine for 90 min and every 5 min after the termination of stress exposure for 15 min.

Evaluation of the development of tolerance: The analgesic effect induced by 10 mg/kg of morphine or by each stress was measured daily for 3 days. The effect was expressed as the area under the curve (AUC) by plotting the increase in response threshold (sec) on the ordinate and the time intervals (min) on the abscissa.

Estimation of $^3$H-leucine incorporation into the TCA-insoluble fraction of mouse brain: Animals were sacrificed by decapitation at various time intervals after morphine or CYH administration (10 mg/kg, i.p.) and immediately after the termination of stress exposure. In the experiment with tolerant animals, they were sacrificed 1 hr after the final morphine treatment or immediately after the final exposure to the stresses. Ten min before sacrifice, mice were injected i.p. with 10 μCi of $^3$H-leucine (NEN, 5 Ci/mmol). After decapitation, the brain was quickly removed and dissected into 3 discrete regions (striatum, cerebellum and remainder) on ice according to the method of Glowinski and Iversen (12). Each brain region was then weighed and homogenized in an adequate volume of 5% trichloroacetic acid (TCA). The homogenate was heated for 10 min at 95°C. After cooling, the homogenate was centrifuged (10,000 rpm for 10 min), and the pellet was washed with 5% TCA, 95% ethanol-ether (1:1, v/v). The final pellet was solubilized in Protosol (NEN) and transferred to a vial containing 10 ml of toluene scintillator. Radioactivity was determined by a liquid scintillation counter (Aloka, Model LSC-601, within a 52–56% range of efficiency) and expressed as dpm/g tissue.

Statistical analysis: Statistical significance was evaluated by Student’s $t$-test.

Results

Pretreatment with CYH did not affect the analgesic effect of morphine. CYH also did not modify the maximum analgesia induced by stresses; however, the disappearance of the analgesic effect was prevented by CYH; the response threshold was heightened at 5 to 15 min after exposure to FS-stress and at 10 and 15 min after exposure to IW-stress compared to the corresponding values of the saline treated control groups (Fig. 1). Thus, the total analgesia calculated as area under the curve (AUC) was significantly enhanced by CYH pretreatment in SIA.

As shown in Fig. 2, daily treatment with morphine or daily exposure to stresses easily developed tolerance to the analgesic effect.

![Fig. 1. Effect of cycloheximide on morphine- and stress-induced analgesia in mice. Cycloheximide (10 mg/kg, i.p.) or saline was injected 2 hr before morphine administration (Mor, 10 mg/kg, i.p.) or exposure to stresses (foot shock: FS or immobilized and water-immersion: IW stress for 30 min). The analgesic effect (response threshold, a cut-off time of 6 sec) was measured by the modified Haffner's method, every 15 min after morphine injection for 90 min or every 5 min after stress exposure for 15 min. ▲, ○, □: Saline treated control groups. △, ●, ■: Cycloheximide treated groups. Each point is the mean±S.E. of 24 to 57 animals. *$P<0.05$, **$P<0.01$, compared with the saline treated control group.](image-url)
Fig. 2. Effect of cycloheximide on the development of tolerance to morphine- and stress-induced analgesia in mice. Daily changes of the analgesic effect induced by morphine or stress exposure was measured for 3 consecutive days. Cycloheximide (10 mg/kg, i.p.) was injected daily 2 hr before morphine injection (10 mg/kg, i.p.) or exposure to stresses. The analgesic effect is expressed as the area under the curve (AUC) that is obtained by plotting the increase in response threshold (sec) on the ordinate and the time intervals (min) on the abscissa as shown in Fig. 1. A, O, □: Saline treated control groups. ▲, ●, ■: Cycloheximide pretreated groups. Each point is the mean±S.E. of 24 to 57 animals.

Pretreatment with CYH 2 hr before every morphine injection or every FS exposure effectively depressed the development of tolerance to the analgesic effect. On the contrary, development of tolerance to IW-SIA was not inhibited by CYH, although the analgesic effect was significantly enhanced on the 1st day (Fig. 2).

The incorporation of 3H-leucine into the TCA-insoluble fraction of mouse brain regions was slightly but significantly depressed by 10 mg/kg of morphine. The peak effect was attained 1 hr after injection and returned to the control level by about 3 hr. A marked inhibition of the incorporation was observed 30 min after injection of 10 mg/kg of CYH, but the inhibitory effect of CYH waned rather quickly and disappeared by 2.5 hr (Fig. 3). No difference was noted between the 3 discrete brain regions in the incorporation of 3H-leucine under the effect of morphine and CYH.

Pretreatment with CYH 2 hr before every morphine injection or every FS exposure completely nullified the inhibitory effect of CYH on the incorporation of 3H-leucine into the TCA-insoluble fraction of mouse brain regions. After i.p. injection of cycloheximide (10 mg/kg), morphine (10 mg/kg) or saline, animals were sacrificed at various time intervals. Ten min before sacrifice, 10 μCi of 3H-leucine was injected i.p. The results are expressed as percent of the incorporation in the saline treated control group. Each point is the mean of 4 to 6 experiments. *P<0.05, **P<0.01, ***P<0.001 compared with the control.

The inhibitory effect of morphine on the incorporation of 3H-leucine into the TCA-insoluble fraction of brain regions, the striatum and the remaining portion except the cerebellum, 1 hr after injection was completely nullified by the pretreatment with CYH. A similar result was obtained in the cerebellum (data not shown). The incorporation of 3H-leucine was not influenced by FS-stress, but markedly suppressed by IW-stress. In contrast with morphine, the effect evoked by the exposure to the stresses in the incorporation of 3H-leucine into brain regions was not influenced by the pretreatment with CYH (Fig. 4).

In morphine tolerant animals which received daily injections of morphine for 3 days, the same dose of morphine failed to inhibit the incorporation of 3H-leucine into brain regions and daily pretreatment with CYH could not modify the morphine effect. On the other hand, in SIA tolerant animals, both stresses showed the same effect as in the saline treated control animals; namely, FS-stress caused no change and IW-stress induced a marked reduction in the incorporation of 3H-leucine into brain regions. Daily pretreatment with CYH failed to induce
any appreciable changes in FS-tolerant animals, but the inhibitory effect was partially weakened in IW-tolerant animals (Fig. 5).

**Discussion**

As it has been reported (1, 13), the analgesic effect of morphine was not modified by the pretreatment with CYH. On the other hand, the short-lasting analgesia induced by stresses was prolonged by CYH pretreatment, and the overall analgesic effect, calculated as AUC, was significantly enhanced.

The difference in the effect of CYH between morphine and SIAs was also observed in the development of tolerance to the analgesic effect. Namely, pretreatment with CYH inhibited the development of tolerance to FS-SIA as well as morphine analgesia; however, the effect of CYH was not found in IW-SIA. The discrepancies between morphine and SIAs coincide well with our previous report (9) and may suggest the differences of the underlying mechanisms for the production of analgesia and also for the development of tolerance to the effects.

These differences were further investigated by the incorporation of $^3$H-leucine into the TCA-insoluble fraction of mouse brain regions. The incorporation of $^3$H-leucine into brain regions was determined 60 min after the final injection of morphine or immediately after the final exposure to the stresses for 3 consecutive days. The incorporation of $^3$H-leucine into brain regions was determined 60 min after the final injection of morphine or immediately after the final exposure to the stresses. For other details, refer to the footnote of Fig. 4. $^*P<0.05$, compared with the matched IW-tolerant group. $^**P<0.01$, compared with the saline treated control group.
the non-specificity of the effect. This also confirms that the incorporation was not changed in FS-SIA, and on the contrary, significantly inhibited in IW-SIA, although the effect was rather weak and short-lasting compared with that of morphine. Thus, the inhibition of the incorporation of $^3$H-leucine into the TCA-insoluble fraction of brain regions, namely, the protein synthesis mechanism, may not directly correlate with the production of the analgesic effect.

This is also evident from the effect of CYH, a potent inhibitor of protein synthesis in eukaryotic cells, on the incorporation of $^3$H-leucine into the brain protein fraction. Pretreatment with CYH, 2 hr before morphine administration, could not modify the analgesic effect of morphine, but it completely reversed the inhibitory effect of morphine on the incorporation of $^3$H-leucine into brain regions observed at the peak of the analgesic effect. Immediately after exposure to stress, when the analgesic effect was maximum, the incorporation of $^3$H-leucine was not changed in FS-SIA and was significantly suppressed in IW-SIA; however, these effects were not modified by CYH pretreatment. These results indicate not only the non-participation of the protein synthesis mechanism in the production of analgesia but also the existence of diverse underlying mechanisms in the processes.

We have previously reported that pretreatment with CYH inhibits the development of acute tolerance which was observed 24 hr after a single dose of morphine, and furthermore, the effect of CYH is strictly dependent on the timing of its administration (13). Thus, it is suggested that the effect of CYH at the initial dose of morphine may affect the process of the development of tolerance to the analgesic effect. The inhibitory effect of morphine on the incorporation of $^3$H-leucine into brain regions was lost in morphine tolerant animals. These facts may indicate the involvement of protein synthesis in the process of tolerance development.

Implication of the protein synthesis mechanism in the development of tolerance to morphine analgesia has been reported (3, 4). However, as far as SIA is concerned, the effect of SIA on the incorporation of $^3$H-leucine in the brain regions of control animals was not altered in SIA-tolerant animals, and the pretreatment with CYH could not affect the development of tolerance to IW-SIA, but significantly inhibited that to FS-SIA. Here, the differences between morphine and SIAs and also between each SIA are demonstrated again, indicating the differences of the underlying mechanisms.

Thus, the protein synthesis mechanism is greatly influenced by morphine and stresses, but we could not demonstrate definite evidence that the mechanism is directly implicated in the production of the analgesic effect and in the development of tolerance to the effect. However, the present experiment revealed that multiple mechanisms are involved in the process of the production of analgesia and tolerance.

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