Full Paper

Underlying Mechanism of Combined Effect of Methamphetamine and Morphine on Lethality in Mice and Therapeutic Potential of Cooling

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Abstract. An increase in polydrug abuse is a major problem worldwide. A previous study showed that coadministration of methamphetamine and morphine induced lethality in rodents and humans. However, the underlying mechanisms by which the lethality is increased by the coadministration of methamphetamine and morphine have not been fully understood. Therefore, the present study was designed to determine the mechanism of increased lethality induced by methamphetamine and morphine. Coadministered methamphetamine and morphine increased the lethality by more than 70% in BALB/c mice. Pretreatment with NMDA-receptor antagonists, such as MK-801 and 3-((R)-2-carboxypiperazin-4-yl) propyl-1-phosphonic acid (CPP), and benzamide [poly(ADP-ribose) polymerase (PARP) inhibitor] significantly attenuated the increased lethality induced by methamphetamine and morphine. Furthermore, the lethal effect induced by methamphetamine and morphine was completely attenuated by immediate cooling after the coadministration of methamphetamine and morphine. It has been reported that methamphetamine-induced neurotoxicity can be blocked by lowering the temperature, and this effect might be mediated by a reduction of release of free radicals. These results suggest that activation of NMDA receptors and PARP play an important role in the increased lethality induced by methamphetamine and morphine.

Keywords: methamphetamine, morphine, toxicity, oxidative stress

Introduction

The increase in substance abuse is a major problem worldwide. Methamphetamine abuse has dramatically increased over the past two decades (1), and the number of teenagers abusing methamphetamine has been increasing in recent years. Acute administration of methamphetamine results in an increase in blood pressure, heart rate, respiration, sweating, and tremors, and it occasionally causes death (2, 3). Therefore, methamphetamine abuse now constitutes a serious health problem that is reflected by the increasing number of emergency room visits by drug abusers (1).

Increasing evidence shows that methamphetamine produces psychosis and damage to monoaminergic (especially dopaminergic) neurons in the central nervous system (2). Methamphetamine-induced neurotoxicity is characterized by a long-lasting depletion of striatal dopamine and serotonin (2). A previous study showed that methamphetamine-induced neurotoxicity is also linked to apoptotic mechanisms (2, 3), such as glutamate and nitric oxide systems, and is a result of the increase in free radical formation and oxidative stress within the striatum (4).

Polydrug abuse is a common phenomenon, and drugs from a wide range of pharmacological classes, such as alcohol, opioids, and sedatives, are frequently combined with psychostimulants, such as amphetamine, methamphetamine, and cocaine. For example, “speedball,” which is a combination of cocaine with opioids, such as heroin, is commonly used to produce a more intensely pleasurable “rush.” In addition, a combination of amphetamine with heroin, a “bombitas”, has also been reported (5). A previous study showed that cocaine-heroin/morphine or methamphetamine-heroin/morphine combinations produce more rewarding and discrimina-
tive stimulus effects in animals than identical doses of cocaine or methamphetamine alone (6 – 10).

Speedball induces serious detrimental effects on physical and mental health and social integration (11). It is well known that in animals, morphine and methamphetamine have pronounced complex effects on the body temperature. In rodents and humans, hyperthermia and hypothermia are the prominent effects of methamphetamine and morphine, respectively. In fact, two fatal cases, which might be related to the coadministration of methamphetamine and morphine, have been reported in humans (12). Funahashi et al. (13) showed that the coadministration of methamphetamine and morphine induces marked hyperthermia rather than methamphetamine alone in mice. Furthermore, it has been noted that lethality was enhanced by methamphetamine and morphine (13). Our preliminary study showed that a combination of 20 mg/kg of methamphetamine, a dose that induces self-injurious behavior, and 20 mg/kg of morphine, the dose which induces hyperlocomotion and antinociceptive effects, increased the lethality by more than 70%. However, the underlying mechanisms by which the coadministration of methamphetamine and morphine increases the lethality and the relationship between these lethal effects and hyperthermia have not been fully understood. The present study was designed to determine the mechanism of lethal effects induced by methamphetamine and morphine. The focus of this study is to elucidate the underlying mechanism between methamphetamine-induced neurotoxicity and methamphetamine and morphine induced lethality, and the relationship between hyperthermia and lethal effects. We also investigated the relative importance of temperature control in preventing the lethal effects induced by methamphetamine and morphine.

Materials and Methods

Animals

Male BALB/c mice (Charles River Japan Inc., Atsugi) weighing 18 – 23 g were used for the experiments. The animals were housed at room temperature 20 – 25°C under a 12 h light-dark cycle (lights on at 8:00 AM). Food and water were available ad libitum. All procedures were conducted in accordance with the guiding principles for the care and use of laboratory animals by the Japanese Pharmacological Society and the Tokyo Women’s Medical University Committee guidelines on animal care and use.

Measurement of behavioral observations and colonic temperature

The behavior (e.g., stereotyped behavior, locomotion, sedation, and ataxia), toxicity, and death of the mice were observed just before and after the administration of methamphetamine and/or morphine at 30, 60, 90, 120, 180, 240, 360, and 480 min and at 24 and 48 h under single-blind conditions. After the administration of methamphetamine and morphine, the mice were placed in individual cages. Methamphetamine and/or morphine were administrated subcutaneously once in an experiment at 10 – 11 AM in a room maintained at 20 – 25°C and 45 ± 5% relative humidity.

The colonic temperature was recorded as follows: A digital thermometer (TD-300; Shibaura Electronics Co., Ltd., Tokyo) attached to a rectal temperature nonstick probe (Thermal sensor; Shibaura Electronics Co., Ltd.) was used to determine the rectal temperature. The probe was inserted into the colon at a constant depth of 2.5 cm and removed after each reading. In the present study, colonic temperature was immediately measured before and after the administration of methamphetamine (2, 8, and 20 mg/kg) and/or morphine (20 mg/kg) at 30, 60, 90, 120, 180, 240, 360, and 480 min and at 24 and 48 h.

In antagonism tests, the following drugs were pretreated with methamphetamine (20 mg/kg) and morphine (20 mg/kg): 30 min for 1 mg/kg each of haloperidol and SCH23390 (i.p.), 30 min for 0.125 – 0.5 mg/kg of MK-801 (i.p.), 30 min for 5 mg/kg of 3-((R)-2-carboxypiperazin-4-yl) propyl-1 phosphonic acid (CPP), 30 min for 100 – 400 mg/kg of N^N-nitro-Larginine methyl ester hydrochloride (L-NAM), 30 min for 25 and 50 mg/kg of 7-nitroindazole (7-NI) (i.p.), and 30 min for 160 – 320 mg/kg of benzamid (i.p.). The time and dose were determined based on previous reports (13 – 20).

Cooling

After the coadministration of methamphetamine and morphine at 0, 30, and 90 min, the mice were individually housed in plastic bottle cages on two separated aluminium boards. Crushed ice was placed between the boards in order to stabilize the temperature of the boards at 4°C. Attempts were made to control colonic temperatures between 25°C and 30°C. Cooling was continued for 330 min after the coadministration of methamphetamine and morphine.

Immunohistochemical analysis

Tissue specimens: Six mice treated with 20 mg/kg of methamphetamine and 20 mg/kg of morphine and the six non-treated control mice were perfused transcardially with 0.01 M phosphate-buffered saline (PBS,
pH 7.4) and then with 10% formalin for 10 min after anesthesia using pentobarbital at a flow rate of 50 ml/min. The heart, liver, and brain were immediately removed; and then they were immersed in 10% formalin overnight, embedded in paraffin wax in a routine manner, and serial (3-μm-thick) sections were prepared.

**Histological examination:** Sections stained with routine hematoxylin and eosin (H&E) stain were used. **Immunohistochemical examination:** Rabbit anti-mouse PARP antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at a dilution of 1:200 was used as primary antibody. The sections were pretreated by microwave irradiation with high power for 5 min at 800 W in 0.01 M citric acid buffer (pH 6.0) and incubated for overnight at 4°C in primary anti-poly(ADP-ribose) polymerase (PARP) antiserum (1:200) after 30 min intrinsic peroxidase blocking using 0.3% H2O2 at room temperature. After incubation, they were immuned in the secondary antiserum containing biotinylated second antibody (DakoCytomation; Glostrup, Denmark) for 30 min and enzyme conjugate for 20 min from the Histmouse-SP Plus kit (Zymed Laboratories, San Francisco, CA, USA). Immunoreactivity was visualized by Simple Stain DAB solution Histofine (Nichirei, Co., Tokyo) for 10 min at room temperature.

**Positive and negative controls:** Follicular centers and interfollicular zone cells of formalin-fixed embedded human tonsil sections were used for checking sensitivity and reproducibility of immunohistochemical staining for PARP. Negative controls were also set up through omission of the primary antibody.

**Counting of immunoreactive cells:** PARP immunoreactive cells were counted in contiguous high power fields (objective ×200) at each square sampling region (500 × 500 μm2) using a microscope (BX50; Olympus, Tokyo). Five areas randomly selected per one mouse were used for the measurement and each average score was calculated.

**Drugs**

The drugs used in the present study were methamphetamine hydrochloride (Dainippon Pharmaceutical Co., Osaka), morphine hydrochloride (Sankyo Co., Tokyo), MK-801 (Sigma Chemical Co., St. Louis, MO, USA), CPP (Tocris Cookson, Ltd., Bristol, UK). L-NAME, 7-NI, benzamide (Wako Pure Chemical Industries, Ltd., Osaka); R- (+)-SCH23390 (Sigma); and haloperidol (Serenace Injection®) (Dainippon Pharmaceutical Co.) were also used. All drugs were dissolved in saline with the exception of benzamide and 7-NI. Benzamide and 7-NI were dissolved in sterile saline containing 1% Tween 80 and 0.5% carboxymethyl-cellulose, respectively. All drugs were administered at a dose of 0.1 ml/10 g body weight.

**Statistical analysis**

Data were expressed as the mean ± S.E.M. χ2 test and the Mann-Whitney U test were used to determine if there was a significant difference for the survival rate and the colonic temperature, respectively. In the pathological study, the Mann-Whitney U test was used to determine if there was a significant difference for the statistical analysis. P<0.05 was considered to be statistically significant.

**Results**

**Measurement of behavioral observations and colonic temperature**

The acute administration of methamphetamine (20 mg/kg) induced significant hyperthermia within 30–120 min, and the colonic temperature subsequently decreased (Fig. 1). Rearing, sniffing, salivation, and self-injurious behavior, such as skin-picking and biting, were induced by 8 and 20 mg/kg of methamphetamine in most of the mice. On the other hand, morphine (20 mg/kg) initially decreased the locomotor activity, while increased locomotor activity was observed after 30 min by the Straub tail reaction. Furthermore a significant hypothermia was observed within 30 min (Fig. 1). Subsequently, hyperlocomotion, Straub tail reaction, and colonic temperature gradually returned to normal at 180 min (Fig. 1).

Methamphetamine plus morphine induced a significant hyperthermia within 30 min (Fig. 1), and potent hyperlocomotion accompanied by the Straub tail reaction was observed. Subsequently, colonic temperature normalized within 60 min, and the temperature was maintained until approximately 240 min after the coadministration of methamphetamine and morphine (Fig. 1). Approximately 60 min after the administration of methamphetamine and morphine, apparent miosis was observed (the pupil changed to pure white). Ataxia was observed in most of the mice 180 min after with the coadministration of methamphetamine and morphine. Approximately 360 min after the coadministration of methamphetamine and morphine, the colonic temperature suddenly began to decrease (Fig. 1), and behavioral disruption was observed. Finally, tremors were observed immediately prior to death. More than 70% of mice were dead within 24 h after the coadministration of methamphetamine and morphine (Fig. 1).

In antagonism tests, MK-801 and CPP did not affect the acute phenotype of behavior induced by methamphetamine and morphine. However, the colonic temperature of the mice treated with MK-801 (0.125
mg/kg) and CPP [MK-801 and CPP itself significantly decreased the colonic temperature (data not shown)] was significantly decreased by more than 6°C within 120 min after the coadministration of methamphetamine and morphine (Fig. 2A). Subsequently, the colonic temperature returned to the control levels within 360 min (Fig. 2A). The increase in lethality induced by methamphetamine and morphine was significantly and almost completely attenuated by MK-801 and CPP (Fig. 2A), and the behavior phenotype of the treated mice normalized in 360 min in comparison with the control mice.

Although L-NAME administration (100 and 400 mg/kg) inhibited the activity of mice in the pretreatment period, hyperactivity and miosis were not inhibited at 30–60 min after the coadministration of methamphetamine and morphine. In comparison with the control, L-NAME induced lower colonic temperature within 30 min in combination with methamphetamine and morphine (Fig. 2A). L-NAME tended to attenuate the lethality induced by methamphetamine and morphine; however, this effect was not significant (Fig. 2A). Seventy percent of mice treated with L-NAME died without normalization of their colonic temperature 48 h after the coadministration of methamphetamine and morphine (Fig. 2A). On the other hand, in comparison with control mice, 7-NI administration in combination with methamphetamine and morphine did not inhibit hyperactivity, miosis, and ataxia and increased colonic temperature within 30 min of administration (Fig. 2B). 7-NI significantly aggravated the lethality induced by methamphetamine and morphine; all the mice died 24 h after the coadministration of methamphetamine and morphine (Fig. 2B).

In the pretreatment period, hypoactivity was observed after administration of benzamide (160 and 320 mg/kg). However, benzamide did not attenuate the hyperactivity, and it dose-dependently decreased the colonic temperature 30 min after the coadministration of methamphetamine and morphine (Fig. 2B). Subsequently, the colonic temperature normalized within 60–90 min (Fig. 2B). Twenty-four hours after the coadministration of methamphetamine and morphine, 320 mg/kg benzamide significantly attenuated the methamphetamine and morphine-induced lethality (Fig. 2B).

Pretreatment of SCH23390 and haloperidol attenuated the hyperactivity induced by methamphetamine and morphine. However, it did not affect ataxia and the higher colonic temperature induced by methamphetamine and morphine. Most mice were dead by 24 h; SCH23390 plus haloperidol significantly aggravated the lethality induced by the coadministration of methamphetamine and morphine (Fig. 2A).

Cooling

The lethal effect induced by methamphetamine and morphine was significantly and almost completely attenuated by immediate cooling after the coadministration of methamphetamine and morphine (Fig. 3). During the cooling period, hyperlocomotion induced by methamphetamine and morphine was not observed. However, the mechanism(s) of hyperlocomotion induced by methamphetamine and morphine could not be determined because it is difficult to determine whether these effects of cooling on the behavior are related to the mechanism(s) of hyperlocomotion itself (e.g., physically, such as masked by freezing). After the cooling period, normal behavior such as grooming,
sniffing, and rearing were observed, and the colonic temperature normalized (Fig. 3). In order to ascertain the effectiveness of cooling for lethality induced by methamphetamine and morphine, we also examined the “golden hour,” which is the time of expectable treatments against the lethality induced by methamphetamine and morphine. The effectiveness of cooling on the lethality induced the coadministration of methamphetamine and morphine was observed after cooling at 30 min, and therapeutical effects of cooling were the same as those of immediate cooling after drug administration (Fig. 3). On the other hand, therapeutic effects of cooling on the lethal effects induced by methamphetamine and morphine were not observed after cooling at 90 min (Fig. 3). Behavioral disruption was observed after administration in a 120-min period, and most mice were dead by 24 h (Fig. 3).

**Immunohistochemical analysis**

The number of PARP-positive cells were significantly increased in cardiac muscle cells and cardiac stromal cells ($P = 0.003$) and in hepatic cells and hepatic stromal cells ($P = 0.003$) of mice treated with methamphetamines and morphine (Fig. 4). The immunoreactivity of PARP was confirmed to be positive in the positive controls and confirmed to be negative in the negative controls. In addition, PARP-positive cells were slightly increased in the striatum; however, these effects were rare as compared with those observed in cardiac muscle cells and cardiac stromal cells and in hepatic cells and hepatic stromal cells of mice treated with methamphetamines and morphine.

**Fig. 2.** Effect of MK-801, CPP, L-NAME, and SCH23390 + haloperidol (A, B) and 7-NI and benzamide (C, D) on the survival rate (A, C) and colonic temperature (B, D) in mice treated with methamphetamine and morphine. Each point represents the mean counts with S.E.M. of 10 animals. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs vehicle control.
Discussion

In the present study, we showed that the coadministration of methamphetamine and morphine increased the lethal effects, and these effects were almost completely attenuated by non-competitive N-methyl-D-aspartate (NMDA)-receptor antagonists such as MK-801 and CPP. These results indicated that the lethal effects induced by methamphetamine and morphine were mediated through the activation of NMDA receptors. Recent studies have demonstrated that oxidative stress including the activation of PARP is involved in methamphetamine-induced neurotoxicity (21) and that methamphetamine-induced increase of radical release was not observed at low temperature (22). Therefore, we also examined the involvement of oxidative stress in the lethal effects induced by methamphetamine and morphine. In the present study, the administration of the PARP inhibitor, benzamide, and cooling significantly attenuated the lethal effects induced by methamphetamine and morphine. These results suggest that oxidative stress is closely related to the lethal effects induced by methamphetamine and morphine. Therefore, our findings may suggest a potential therapy for certain forms of drug-induced toxicity.

It has been well established that NMDA-receptor antagonists such as MK-801 have neuroprotective properties (23). The mechanism of action of MK-801 is complex; in addition to blocking NMDA receptors, MK-801 can also enhance both dopaminergic and serotonergic neurotransmission (24 – 26). In the present study, CPP (a competitive NMDA-receptor antagonist) almost completely attenuated the increased lethality induced by methamphetamine and morphine. These results strongly suggest that the coadministration of methamphetamine and morphine-induced lethality in mice is mediated through the activation of NMDA receptors.

The NMDA-receptor activation leads to the increased production of reactive species (27), particularly through the production of nitric oxide (NO) via the coordinated action of nitric oxide synthase (NOS) (28). In the present study, we showed that blocking the endogenous NO production by systemic injection of L-NAME (a non-selective NOS inhibitor) slightly, but not significantly, suppressed coadministered methamphetamine and morphine-induced lethality. This indicated that NO may be partially related to the increased lethality induced by methamphetamine and morphine. On the other hand, previous studies utilizing neuronal cells in primary culture have indicated that the activation of PARP, an enzyme involved in DNA plasticity-related phenomena, is an early event in glutamate-induced neurotoxicity (29) and that inhibitors of PARP, including benzamide, have a protective effect against methamphetamine-induced neurotoxicity (19, 30). The present study interestingly demonstrated that benzamide could partially, but significantly, attenuate the coadministered methamphetamine and morphine-induced lethality. It is possible that methamphetamine not only kills neurons by the direct production of free radicals but also by triggering a mitochondria-dependent induction of apoptotic cascades (31). Therefore, coadministered methamphetamine and morphine-induced lethality was accompanied by activation of apoptotic pathways as a toxic response. In addition, the activation of PARP results in a massive depletion in cellular energy that is stored in the form of NAD$^+$ and ATP (29, 32). In the present study, the
increase in lethality induced by methamphetamine and morphine was associated with prolonged hypothermia. Therefore, the prolonged hypothermia induced by methamphetamine and morphine may be related to energy depression. In fact, the pathological study showed that PARP immuno-reactivity in cardiac muscle cells and hepatic cells was significantly increased in coadministration of methamphetamine and morphine. Therefore, the potent toxicity to some organ (e.g., heart and/or liver) might involve the main lethal effect by coadministered methamphetamine and morphine in treated mice.

Hypothermia may also be related to the underlying mechanisms by which the drugs used in the present study could attenuate coadministered methamphetamine and morphine-induced lethality in mice. The potency order of cooling or drugs with respect to the attenuating effects for decreasing the lethality induced by methamphetamine and morphine was consistent with the effectiveness of the cooling effect in combination with methamphetamine and morphine (cooling > CPP > MK-801 > benzamide > L-NAME). These results indicate that cooling is beneficial because death, which is induced by methamphetamine and morphine, is avoided. It has been reported that methamphetamine-induced hyperthermia and neurotoxicity can be blocked by lowering ambient temperature to 5°C, which is related to reduction in free radical release (33, 34). These results support the hypothesis that NO is involved in the increased lethality induced by methamphetamine and morphine.

To the best of our knowledge, there have been no reports investigating the effectiveness of the cooling treatment in the “golden hour.” In the present study, the effectiveness of the cooling treatment for lethality induced by methamphetamine and morphine was observed after 30 min, but not 90 min. These results suggest the existence of the “golden hour” for the treatment of toxicity induced by the coadministration of methamphetamine and morphine.

Dopamine-receptor antagonists have been previously shown to protect against damage to dopaminergic nerve
The combination of SCH23390 (D₁-receptor antagonist) or haloperidol (D₂-receptor antagonist) with methamphetamine-induced neurotoxicity. However, these results strongly indicate that the mechanisms of neurotoxicity induced by methamphetamine were clearly different from those of increased lethality induced by methamphetamine and morphine. Therefore, noradrenaline and/or serotonin (2), or activation of arachidonic cascade, which might increase the release of free radical (39, 40), might be involved in the increased lethality-induced by methamphetamine plus morphine. Further examination is needed to determine why dopamine receptor antagonists and nNOS inhibitor aggravated the increased lethality induced by methamphetamine and morphine.

In summary, we demonstrated that the administration of NMDA-receptor antagonists, a PARP inhibitor, and cooling significantly attenuated the co-administration of methamphetamine and morphine-induced lethality. The present study indicates that oxidative stress is involved in co-administered methamphetamine and morphine-induced lethality. We also demonstrated the existence of the “golden hour” for treatment of the lethality induced by methamphetamine and morphine. These findings may help in improving the medical treatment to individuals indulging in the abuse of methamphetamine and morphine.

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