to potentiate cocaine induced-hyperactivity. Our data suggest that thioperamide, at least at 10 mg/kg, increases cocaine-induced locomotion through the combination of pharmacokinetic effects and the blockade of H_3 receptors located on non-histaminergic neurons.

Keywords Cocaine · Histamine · Thioperamide · Immepip · A-331440 · H_3 receptor · Locomotion · Knockout mouse

Introduction

Several studies have demonstrated the role of histamine neurotransmission in various behavioral functions. Histaminergic neurons originate from the tuberomamillary nucleus of the posterior hypothalamus and project to most areas of the brain. Histamine is synthesized from L-histidine by the enzyme histidine decarboxylase (HDC) and acts in the brain through three types of G-protein-coupled receptors: the histamine H_1, H_2, and H_3 receptors. H_1 and H_2 receptors are essentially postsynaptic excitatory receptors. In contrast, H_3 receptors are coupled to $G_{i/o}$ proteins and are mainly inhibitory receptors. They were originally described as presynaptic autoreceptors located on histaminergic fibers, as their activation produces an inhibition of histamine release and synthesis. However, subsequent studies have found that the majority of H_3 receptors are inhibitory heteroreceptors located on non-histaminergic fibers. Their activation can inhibit the synthesis and release of various neurotransmitters [namely, gamma-aminobutyric acid (GABA) and dopamine] via G-protein-mediated inhibition of presynaptic calcium channels (Brown et al. 2001). Lesion studies have also revealed that the majority of H_3 receptors in the striatum are located postsynaptically on GABAergic medium-sized spiny neurons and are coupled to the inhibition of adenlyate cyclase (Pollard et al. 1993; Ryu et al. 1994).

In both humans and animals, H_3 receptors are particularly abundant in the ventral striatum (Pollard et al. 1993; Goodchild et al. 1999), a brain region that plays a major role in the psychomotor and rewarding effects of psychostimulants (Ikemoto 2007). Consistent with this localization, the H_3 receptor inverse agonists thioperamide and clobenpropit potentiate methamphetamine-induced elevations in dopamine levels in the nucleus accumbens and self-administration of methamphetamine in rats (Munzar et al. 2004). Thioperamide also enhances place preference and locomotion induced by cocaine in mice (Brabant et al. 2005; Brabant et al. 2006). Moreover, this compound increases the locomotor effects of SKF-38393 and quinpirole in reserpinized mice (Ferrada et al. 2008). Finally, clobenpropit and thioperamide potentiate the subjective effects of methamphetamine and cocaine in rats (Munzar et al. 1998, 2004; Mori et al. 2002; Campbell et al. 2005). In contrast to these studies, H_3 inverse agonists decrease the locomotor effects of methamphetamine and amphetamine and these effects have been reported to result from an increased release of brain histamine produced by the blockade of H_3 autoreceptors (Clapham and Kilpatrick 1994; Morisset et al. 2002; Akhtar et al. 2006). In agreement with that hypothesis, systemic injections of histidine, leading to an increased concentration of brain histamine, reduce the stimulant effects of methamphetamine in mice (Itoh et al. 1984). Moreover, knockout mice lacking the enzyme histidine decarboxylase (HDC KO) are deficient in histamine and show increased methamphetamine-induced hyperactivity (Kubota et al. 2002).

Our previous studies have found that thioperamide strongly potentiates locomotor activity induced by different doses of cocaine (8 and 24 mg/kg) in C57BL/6J mice but did not enhance or reveal stereotypies produced by this psychostimulant. We have suggested that the effects of thioperamide on cocaine-induced locomotion result from increased dopaminergic activity produced by the blockade of H_3 heteroreceptors located in the ventral striatum (Brabant et al. 2005, 2006). However, the involvement of H_3 receptors in these effects of thioperamide has not been demonstrated yet. Moreover, thioperamide increases histamine in the brain through the blockade of H_3 autoreceptors (Arrang et al. 1983), and central injections of histamine have been found to activate the mesolimbic system and to increase locomotor activity (Kalivas 1982; Fleckenstein et al. 1993; Chiavegatto et al. 1998). These results raise the possibility that the potentiating effects of thioperamide are mediated by an increased release of histamine. Therefore, the purpose of the present study was to examine the involvement of histamine and H_3 receptors in the ability of thioperamide to enhance cocaine-induced locomotion. In a first set of experiments, we investigated whether immepip, a highly selective H_3 agonist, could reverse the effects of thioperamide on cocaine-induced hyperactivity. Furthermore, A-331440, a non-imidazole based H_3 inverse agonist, was tested on the locomotor effects of cocaine (Hancock et al. 2004). We also measured cocaine plasma concentrations after the injections of thioperamide and cocaine to investigate potential pharmacokinetic interactions between these drugs. Finally, in order to examine whether thioperamide enhances cocaine-induced hyperactivity through an increased release of histamine produced by the blockade of H_3 autoreceptors, this compound was tested on the locomotor effects of cocaine in HDC KO mice. H_3 receptors display high constitutive activity and spontaneous activation in the absence of histamine (Morisset et al. 2000), suggesting that thioperamide, acting as an inverse agonist, may decrease the activity of these receptors in HDC KO mice. However, these effects would not lead to an increased release of histamine in these mice because they

lack HDC (Moreno-Delgado et al. 2006). Consequently, if the potentiating effects of thioperamide on cocaine-induced hyperlocomotion are mediated through presynaptic H_3 autoreceptors by increasing histamine release, it would be expected that the effects of thioperamide would not be observed in HDC KO mice.

Materials and methods

Animals

In all experiments, male mice (22–29 g) were 9–12 weeks old and housed individually in transparent polycarbonate cages (*L* 26 × *W* 40.5 × *H* 20 cm) with pine sawdust bedding. Food (standard pellets, Carfil Quality BVDA, Oud-Turnhout, Belgium) and water were available ad libitum for the whole experiment. The animal room was maintained on a 12:12 h light–dark cycle (lights on at 0800 hours) with an ambient temperature of 19–22°C. All experimental procedures were carried out during the light period of the light/dark cycle, between 8 A.M. and 2 P.M. In experiments 1–11, a total of 581 C57BL/6J mice were purchased from Janvier (Schaijk, The Netherlands) or from Jackson laboratory (Bar Harbor, ME, USA). In experiment 12, 36 histidine decarboxylase knockout mice (HDC KO mice) and 36 wild-type mice were used. The HDC KO mice were generated according to previously described procedures (Ohtsu et al. 2001) and maintained on an isogenic 129/Sv genomic background. In experiment 12, isogenic wild-type 129/Sv mice served as controls for HDC KO mice, and the two genotypes were simultaneously evaluated. Genotypes were verified using polymerase chain reaction of tail-tip DNA. All experimental procedures were carried out in accordance with the standards of care and use of laboratory animals laid down by the European Communities Council (Directive no. 86/609/EEC, 11/24/86). Protocols were reviewed and approved by the Animal Care Committee of Liège University.

Pharmacological treatments

Cocaine hydrochloride (Belgopia, Louvain-La-Neuve, Belgium), thioperamide maleate, A-331440 (Sigma-Aldrich, Bornem, Belgium), and immepip dihydrobromide (Tocris, Bristol, UK) were dissolved in isotonic saline solution (0.9% NaCl vehicle) and administered intraperitoneally (i.p.) in a volume of 0.01 ml/g.

Measurement of locomotor activity

Locomotor activity was measured with ten individual test chambers, each one comprising a square enclosure made from 0.5-cm clear acrylglas panels without base (20.5 × 20.5 × 28.5 cm height). An enclosure was placed on a square plate of 0.5-cm gray acrylglas, which served as floor, and a removable, perforated, clear acrylglas plate that served as lid. Ambulatory activity was measured by a pair of infrared light-beam sensors located on each side of the enclosure at a height of 2 cm and spaced 6.5 cm from each end of a side and was recorded on a single PC. A mouse had to traverse the full distance (at least 6.5 cm) between two parallel beams for each locomotor count. Each chamber was encased in a sound-attenuating shell (100 × 90 × 150 cm height), ventilated, illuminated by a non-heating 9-W white light (350 lm), and maintained at 21–23°C. A one-way window on each shell door allowed direct visual surveillance.

Procedure

Experiments 1–10 examined the locomotor effects of H_3 receptor ligands alone and on the effects of cocaine (8 mg/kg, i.p.) in C57BL/6J mice. Animals received a saline injection and were habituated to the testing chambers for 60 min. The following day, mice were randomly allocated to the pharmacological conditions described below (procedures are summarized in Table 1).

In the first experiment (dose-response curve of cocaine-induced locomotion), five groups of mice (n=12) received an injection of saline followed 5 min later by saline, 4, 8, 16, or 24 mg/kg cocaine and were immediately placed into the testing chambers for 60 min.

Experiment 2 examined the effects of thioperamide on locomotion. Three groups of mice (n=10) received an injection of saline, 10 or 20 mg/kg thioperamide followed 5 min later by a saline injection, and were immediately placed into the testing chambers for 60 min.

Experiment 3 was designed to identify the optimal time interval for thioperamide to increase cocaine-induced activity. Three groups of mice (n=12) received two i.p. injections before a third injection of cocaine (8 mg/kg). The first group of mice received two saline injections, 25 min apart. The second group received 10 mg/kg thioperamide, followed 25 min later by a saline injection, whereas the third group received the same injections but in the reverse order. Five minutes after the second injection, all mice were injected with cocaine and placed into the testing chambers for 60 min. Since the results of experiment 3 showed that thioperamide exerted a maximal effect when injected 5 min before cocaine, in the fourth experiment, four groups of mice (n=10) received an injection of saline, 2.5, 5, or 10 mg/kg thioperamide 5 min before receiving cocaine. Mice were then immediately placed into the test chambers for 60 min.

Experiments 5 and 6 investigated the effects of immepip on spontaneous locomotion and cocaine-induced activity.

Table 1 Experimental protocols used to asses the action of immepip, thioperamide, and the combination of these drugs on spontaneous locomotion and on cocaine-induced hyperactivity (8 mg/kg) in C57BL/6J mice

First injection	Second injection (25 min after the first injection)	Third injection (30 min after the first injection)
Experiment 1: dose–response curve of cocaine-induced locomotion		
–	Saline	Saline
–	Saline	Cocaine 4 mg/kg
–	Saline	Cocaine 8 mg/kg
–	Saline	Cocaine 16 mg/kg
–	Saline	Cocaine 24 mg/kg
Experiment 2: action of thioperamide on spontaneous locomotion		
–	Saline	Saline
–	Thioperamide 10 mg/kg	Saline
–	Thioperamide 20 mg/kg	Saline
Experiment 3: effect of time interval of thioperamide injection on cocaine-induced locomotion		
Saline	Saline	Cocaine 8 mg/kg
Thioperamide 10 mg/kg	Saline	Cocaine 8 mg/kg
Saline	Thioperamide 10 mg/kg	Cocaine 8 mg/kg
Experiment 4: action of different doses of thioperamide on cocaine-induced locomotion		
–	Saline	Cocaine 8 mg/kg
–	Thioperamide 2.5 mg/kg	Cocaine 8 mg/kg
–	Thioperamide 5 mg/kg	Cocaine 8 mg/kg
–	Thioperamide 10 mg/kg	Cocaine 8 mg/kg
Experiment 5: action of immepip on spontaneous locomotion		
Saline	–	Saline
Immepip 10 mg/kg	–	Saline
Immepip 20 mg/kg	–	Saline
Experiment 6: action of immepip on cocaine-induced locomotion		
Saline	–	Cocaine 8 mg/kg
Immepip 10 mg/kg	–	Cocaine 8 mg/kg
Immepip 20 mg/kg	–	Cocaine 8 mg/kg
Experiment 7: action of immepip on the potentiation of cocaine-induced locomotion produced by 10 mg/kg thioperamide		
Saline	Saline	Cocaine 8 mg/kg
Saline	Thioperamide 10 mg/kg	Cocaine 8 mg/kg
Immepip 10 mg/kg	Thioperamide 10 mg/kg	Cocaine 8 mg/kg
Immepip 20 mg/kg	Thioperamide 10 mg/kg	Cocaine 8 mg/kg
Additional experiment testing the action of immepip on the potentiation of cocaine-induced locomotion produced by 2.5 mg/kg thioperamide		
Saline	Saline	Cocaine 8 mg/kg
Saline	Thioperamide 2.5 mg/kg	Cocaine 8 mg/kg
Immepip 10 mg/kg	Thioperamide 2.5 mg/kg	Cocaine 8 mg/kg

All drugs were injected via the intraperitoneal route.

Immepip produces reliable pharmacological effects 30 min after i.p. injection (Lamberty et al., 2003) and was therefore injected 30 min before cocaine. In experiment 5, three groups of mice (n=9) received saline, 10 or 20 mg/kg immepip followed 30 min later by a saline injection. Immediately after the second injection, mice were placed into the testing chamber for 60 min. Conditions for experiment 6 were identical, except that the three groups of mice (n=9–11) received an injection of cocaine instead of saline (second injection).

In experiment 7, four groups of mice (n=13–19) were tested to determine the role of H_3 receptors in the ability of thioperamide to increase cocaine-induced locomotion. Thirty minutes before testing, mice were injected with saline or immepip (10 or 20 mg/kg). Five minutes before testing, mice received a second injection of saline or 10 mg/kg thioperamide. Finally, all mice were injected with 8 mg/kg cocaine and then immediately placed into the test chambers for 60 min. An additional independent experiment was conducted to test whether 10 mg/kg immepip could abolish the potentiation of the locomotor effects of cocaine produced by 2.5 mg/kg thioperamide. In that experiment, mice were randomly divided into three groups (n=17–18). Thirty minutes before testing, they were injected with saline or 10 mg/kg immepip. Five minutes before testing, mice received a second injection of

saline or 2.5 mg/kg thioperamide. Finally, all mice were injected with cocaine (8 mg/kg) immediately before the 60-min test session.

The effect of the H_3 inverse agonist A-331440 on spontaneous locomotor activity was studied in experiment 8. Six groups of mice (n=10) were injected with saline, 2.5, 5, 10, 20, or 40 mg/kg A-331440 (i.p.), and 5 min later with saline. Mice were then immediately placed into the test chambers for 60 min. The dose range used in our experiments was similar to doses of A-331440 that reduced body weight in C57BL/6J mice but not in knockout mice lacking H_3 receptors (Hancock et al. 2004; Hancock and Brune 2005). Experiments 9 and 10 investigated the effects of A-331440 on cocaine-induced hyperlocomotion. Preliminary studies have shown that A-331440 exhibited a higher brain/plasma ratio than thioperamide and rapidly penetrates the blood brain barrier (Hancock et al. 2004). Therefore, experiment 9 tested A-331440 on cocaine-induced locomotion using a time interval (5 min) shown to be optimal for thioperamide to potentiate the locomotor effects of cocaine. Five groups of mice (n=12–13) were injected with saline 2.5, 5, 10, or 20 mg/kg A-331440 (i.p.) 5 min before the cocaine injection (8 mg/kg). They were then immediately placed in the test chambers for 60 min. Experiment 10 was designed in the same way as experiment 9, except that A-331440 was injected 60 min before cocaine. This time interval was chosen because high brain concentrations of A-331440 are achieved 1 h after systemic injection (Hancock et al. 2004).

Plasma cocaine concentrations after the injection of thioperamide were assessed in experiment 11. Eight groups of C57BL/6J mice (n=6) were used. Mice were first injected with saline or thioperamide (10 mg/kg, i.p.), and 5 min later with cocaine (8 mg/kg, i.p.). Mice were then decapitated 2.5, 5, 20, or 60 min after the second injection, and trunk blood was collected. Blood samples were analyzed for cocaine concentrations as described below.

In experiment 12, thioperamide was administered in HDC KO mice and wild-type 129/Sv counterparts 30 min before cocaine because thioperamide markedly increases brain histamine 30 min after i.p. administration (Itoh et al. 1991). HDC KO and wild-type mice were habituated daily (three times) to the testing chambers during 60 min after a saline injection because HDC KO mice show reduced locomotion in a novel open-field but normal habituation to a novel environment (Brabant et al. 2007b). On day 4, mice of each genotype were randomly divided into four groups (n=8–9) and received an injection of saline or thioperamide (10 mg/kg, i.p.) followed 30 min later by an injection of saline or cocaine (8 mg/kg, i.p.). Immediately after the second injection, mice were confined in the testing chambers for 60 min.

Cocaine quantification

At the time points defined in the procedure (experiment 11), mice were killed by decapitation, and trunk blood was collected in 50 ml polypropylene conical tubes containing sodium fluoride (2.5 mg) and potassium oxalate (2 mg). The blood samples were then centrifuged at 1,200×g for 20 min. Plasma was subsequently carefully separated from packed red blood cells, placed into sterile 1.5-ml Eppendorf microcentrifuge tubes, and stored at −80°C until analysis. Cocaine was assayed by reversed phase high-pressure liquid chromatography with ultraviolet detection at 235 nm (Jatlow and Nadim 1990).

Statistical analysis

In experiments 1–10, locomotor activity counts were analyzed using two-way mixed-model analysis of variance (ANOVA), with the drug treatment defined as a between-subject factor and the time course of the session as a within-subject factor. Our previous studies showed that the locomotor effects of cocaine peaked during the first 20 min after 8 mg/kg cocaine (i.p.) in C57BL/6J and 129/Sv mice (Brabant et al. 2006, 2007b). Therefore, total locomotion for the first 20 min was also analyzed independently with a one-way ANOVA, with the drug treatment as a between-subject factor. In order to further compare the effects of thioperamide and A-331440 on cocaine-induced hyperactivity in C57BL/6J mice, ANOVA-based measures of effect size (η^2) were calculated in experiments 3, 4, 9, and 10 (Glantz, 1997).

Plasma cocaine concentrations from experiment 11 were analyzed using a two-way ANOVA with thioperamide and the time course (four time points with independent groups) as between subject factors. Areas under the time vs concentration curve (AUC) between time zero and 60 min were determined (WinNonLin 5.0.1, Pharsight, Mountain View, CA). AUC's and C_{max} were compared using t tests.

In experiment 12, data from each genotype were analyzed independently using a three-way mixed-model ANOVA with thioperamide and cocaine defined as between-subject factors and the time course of the session as a within-subject factor. Total activity for the first 20 min was also analyzed independently with two-way ANOVAs, with thioperamide and cocaine defined as between-subject factors.

Where necessary, square root transformations normalized raw data prior to ANOVA, more nearly meeting the assumption of homogeneity of variances (following a significant Leven's test). For the sake of clarity, means of the raw values are presented in the graphs. Relevant between-mean differences were assessed via the Newman–Keuls test (Glantz 1997). Significance was always set at P<0.05.

Results

Experiment 1: dose–response curve of cocaine-induced locomotion in C57BL/6J mice

Figure 1 shows that doses of cocaine between 4 and 24 mg/kg dose-dependently increase locomotor activity in C57BL/6J mice. The two-way ANOVA performed on the scores for the whole session revealed a significant effect of cocaine ($F_{4,55}$=47.132, P<0.0001), a significant effect of the time course ($F_{5,275}$=91.132, P<0.0001) and a significant interaction between these factors ($F_{20,275}$=6.688, P<0.0001). The one-way ANOVA for the first 20 min also indicated a significant effect of cocaine ($F_{4,55}$=37.064, P<0.0001).

Experiment 2: effects of thioperamide on spontaneous locomotion

The effects of thioperamide on spontaneous locomotor activity are shown in Fig. 2A–C. The two-way ANOVA performed on the locomotor scores for the whole session revealed a significant effect of the time course ($F_{5,135}$= 63.554, P<0.0001) but no main effect of thioperamide and no significant interaction between these factors. However, thioperamide at 20 mg/kg produced hypoactivity during the first 20 min ($F_{2,27}$=5.438, P<0.01; Fig. 2C).

Experiment 3: effects of the time interval between the thioperamide and cocaine injections

When injected 5 or 30 min before cocaine, 10 mg/kg thioperamide potentiated cocaine-induced hyperactivity (Fig. 2D–F). The two-way ANOVA computed over the whole 60-min session indicated a significant group effect ($F_{2,34}$=19.077, P<0.0001, η^2=0.528), a significant effect of the time course ($F_{5,170}$=90.949, P<0.0001), and a significant interaction between these factors ($F_{10,170}$=2.546, P<0.01). Thioperamide injected 5 min before cocaine produced the strongest potentiation of cocaine-induced hyperactivity. However, activity of thioperamide-treated animals was not statistically different at any time point (Fig. 2D). The analysis of the first 20 min showed a significant effect of the group ($F_{2,34}$=10.583, P<0.001, η^2= 0.383), but there was no difference between thioperamide-treated mice (P=0.110; Fig. 2F).

Experiment 4: effects of different doses of thioperamide on cocaine-induced hyperactivity

Thioperamide at 5 and 10 mg/kg potentiated the locomotor effects of cocaine across the entire 60-min session (P<0.05, Fig. 2G). The two-way ANOVA computed over the whole 60-min session yielded a significant effect of thioperamide ($F_{3,36}$=5,584, P<0.005, η^2=0.317), a significant effect of the time course ($F_{5,180}$=59.746, P<0.0001) but no

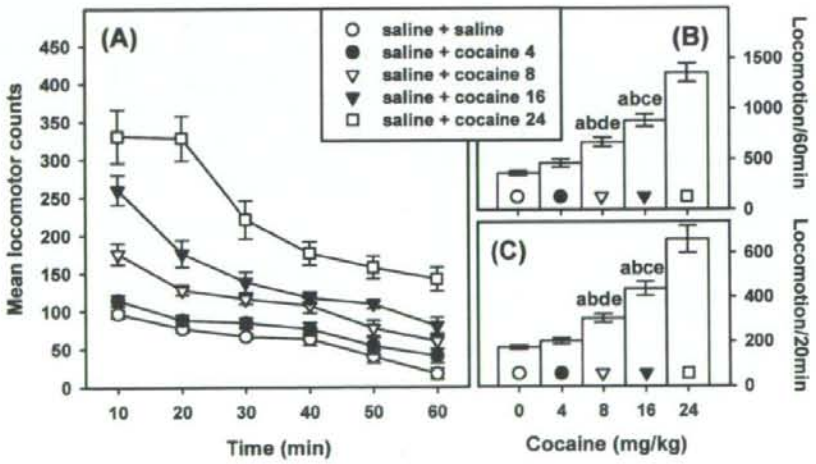

Fig. 1 Dose–response curve of the locomotor effects of cocaine (4–24 mg/kg, i.p.) in C57BL/6J mice. Locomotion is expressed as mean (±SEM) number of locomotor counts. **A** Time course of locomotion in 10-min intervals. Locomotion during the whole session and during the first 20 min is represented in **B** and **C**, respectively. *a* Significantly different from the group injected with saline alone; *b* significantly different from the group injected with 4 mg/kg cocaine; *c* significantly different from the group injected with 8 mg/kg cocaine; *d* significantly different from the group injected with 16 mg/kg cocaine; *e* significantly different from the group injected with 24 mg/kg cocaine

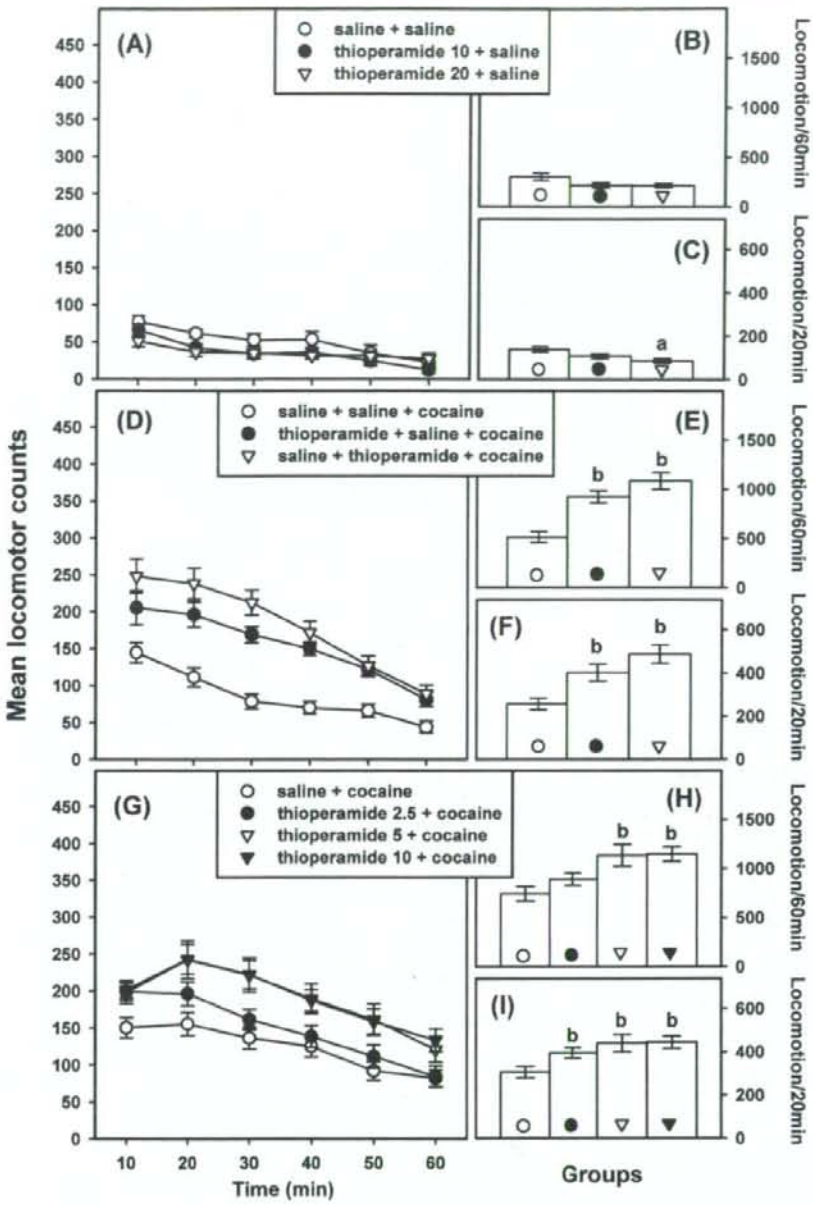

Fig. 2 Action of thioperamide on spontaneous locomotion and cocaine-induced hyperactivity (8 mg/kg, i.p.) of C57BL/6J mice. Locomotion is expressed as mean (±SEM) number of locomotor counts. **A**, **D**, **G** Time course of locomotion in 10-min intervals. **B**, **E**, **H** Total activity for the whole session. **C**, **F**, **I** Total activity for the first 20 min of the session. *a* Significantly different from the group injected with saline alone; *b* value significantly different from the group injected with cocaine alone

significant interaction between these factors. The one-way ANOVA for the first 20 min yielded a significant effect of thioperamide ($F_{3,36}$=4.466, P<0.01, η^2=0.271). During that time interval, all doses of thioperamide significantly potentiated cocaine-induced locomotion (P< 0.05; Fig. 2I).

Experiment 5: effects of immepip on spontaneous locomotion

Immepip at 10 and 20 mg/kg did not significantly affect locomotion (data not shown). The two-way ANOVA for the whole session revealed a significant effect of the time

course ($F_{5,120}$=77.731, P<0.0001) and a significant immepip by time course interaction ($F_{10,120}$=1.931, P<0.05) but no significant effect of immepip. Despite the significant interaction, post-hoc comparisons failed to detect significant differences between groups for any of the time intervals. Moreover, the one-way ANOVA for the first 20 min yielded no significant effect of immepip.

Experiment 6: effects of immepip on cocaine-induced hyperactivity

Immepip at 10 and 20 mg/kg did not significantly alter cocaine-induced hyperactivity (data not shown). The two-way ANOVA computed over the 60-min session revealed a significant effect of the time course ($F_{5,135}$=73.210, P< 0.0001). The main effect of immepip and the immepip by time course interaction were not significant. Moreover, the one-way ANOVA calculated on the data from the first 20 min yielded no significant effect.

Experiment 7: effects of immepip on the potentiation of cocaine-induced hyperactivity by thioperamide

Immepip reduced the action of 10 mg/kg thioperamide on cocaine-induced locomotion during the first 30 min of the session (Fig. 3A–C). The two-way ANOVA for the whole session yielded a significant effect of the drug treatment ($F_{3,65}$=5.507, P<0.005), a significant effect of the time course ($F_{5,325}$=121.216, P<0.0001), and a significant interaction between these factors ($F_{15,325}$=5.832, P< 0.0001). The one-way ANOVA computed on locomotion for the first 20 min also revealed a significant effect of the drug treatment ($F_{3,65}$=3.838, P<0.05).

An additional experiment was carried out to test whether 10 mg/kg immepip could block the potentiation of the locomotor effects of cocaine produced by 2.5 mg/kg thioperamide (Fig. 3D–F). Two-way ANOVA for the whole session indicated a significant group effect ($F_{2,50}$=33.191, P<0.0001), a significant effect of the time course ($F_{5,250}$=

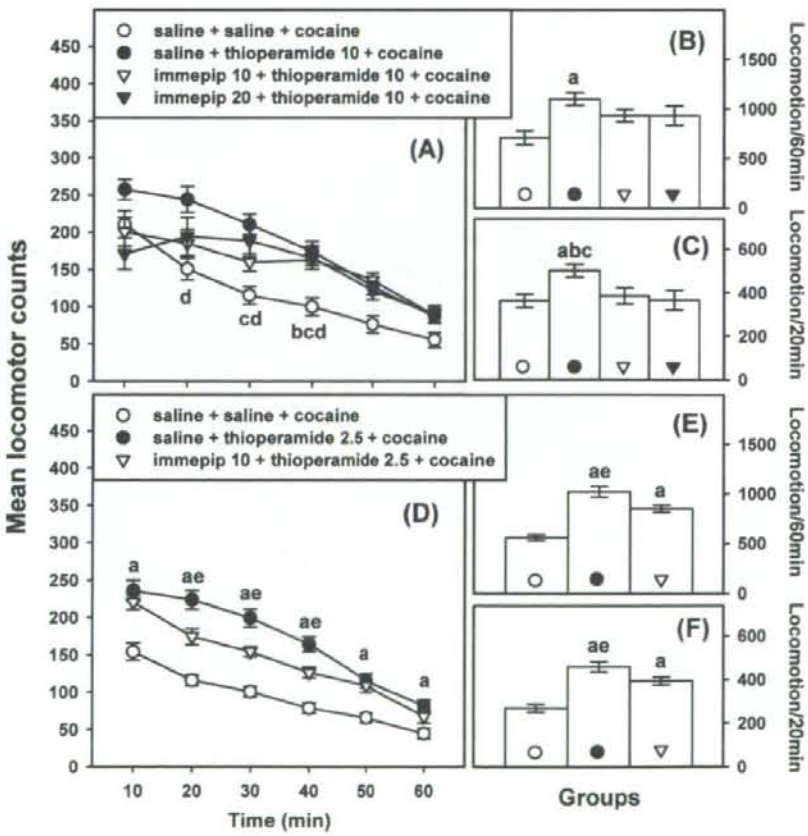

Fig. 3 Effects of immepip on the potentiation of the locomotor effects of cocaine (8 mg/kg, i.p.) induced by thioperamide in C57BL/6J mice. Locomotion is expressed as mean (+SEM) number of locomotor counts. **A**, **D** Time course of locomotion in 10-min intervals. **B**, **E** Total activity recorded during the whole session. **C**, **F** Total activity for the first 20 min of the session. *a* Significantly different from the group injected with cocaine alone; *b* Significantly different from the group injected with 10 mg/kg immepip, 10 mg/kg thioperamide, and cocaine; *c* significantly different from the group injected with 20 mg/kg immepip, 10 mg/kg thioperamide and cocaine; *d* significantly different from the group injected with 10 mg/kg thioperamide and cocaine; *e* significantly different from the group injected with 10 mg/kg immepip, 2.5 mg/kg thioperamide, and cocaine

33.191, $P<0.0001$), and a significant interaction between these factors ($F_{10,250}=4.370$, $P<0.0001$). Post-hoc comparisons indicated that immepip reduced the ability of 2.5 mg/kg thioperamide to increase cocaine-induced hyperactivity during the second, third, and fourth 10-min intervals of the session. The remaining time intervals were unaffected by immepip (Fig. 3D). The one-way ANOVA for the first 20 min yielded a significant group effect ($F_{2,50}=23.735$, $P<0.0001$).

Experiment 8: effects of A-331440 on spontaneous locomotion

Experiment 8 showed that A-331440 did not affect spontaneous locomotion at doses between 2.5 and 20 mg/kg (data not shown). Two-way ANOVA for the whole session revealed a significant group effect ($F_{5,54}=2.667$, $P<0.05$), a significant effect of the time course ($F_{5,270}=99.928$, $P<0.0001$) but no interaction between these factors ($F_{25,270}=0.886$, $P=0.623$). Over the whole session, 40 mg/kg A-331440 significantly decreased locomotion ($P<0.05$). This dose also caused hypoactivity during the first 20 min of the session ($F_{5,54}=3.343$, $P<0.05$) and was thus not tested on cocaine-induced locomotion.

Experiment 9: effects of A-331440 on cocaine-induced hyperactivity injected 5 min before the cocaine injection

A-331440 injected 5 min before cocaine did not significantly increase the locomotor effects of cocaine (data not shown). The two-way ANOVA computed over the 60-min session revealed a significant effect of the time course ($F_{5,295}=103.089$, $P<0.0001$), a significant interaction between the time course and A-331440 ($F_{20,295}=2.262$, $P<0.01$) but no main effect of A-331440 ($F_{4,59}=0.367$, $P=0.831$, $\eta^2=0.024$). The significant interaction resulted from the scores of mice treated with 20 mg/kg A-331440 and cocaine that tended to be lower than those of mice treated with cocaine alone during the last 10-min interval ($P=0.071$). The one-way ANOVA for the first 20 min was not significant ($F_{4,59}=0.789$, $P=0.536$, $\eta^2=0.050$).

Experiment 10: effects of A-331440 on cocaine-induced hyperactivity injected 60 min before the cocaine injection

When injected 60 min before cocaine, A-331440 did not significantly potentiate cocaine-induced hyperlocomotion (data not shown). The two-way ANOVA computed over the whole 60-min session yielded a significant effect of the time course ($F_{5,275}=732.070$, $P<0.0001$). Neither the main effect of the A-331440 treatment nor the A-331440 by time course interaction were significant ($F_{4,55}=0.716$, $P=0.584$, $\eta^2=0.049$; $F_{20,275}=0.639$, P=0.881, respectively). Post-hoc comparisons did not detect significant differences between groups for any of the time intervals. The one-way ANOVA for the first 20 min was not significant ($F_{4,55}=1.809$, $P=0.140$, $\eta^2=0.116$). Over that time interval, A-331440 produced a slight non-significant increase of cocaine-induced locomotion. Mice treated with cocaine alone displayed the lowest locomotor scores (252.83±21.61 locomotor counts) and mice treated with 5 mg/kg A-331440 and cocaine displayed the highest scores (338.25±22.4 locomotor counts). However, post-hoc comparisons did not reveal any significant differences between groups ($P>0.099$).

Experiment 11: plasma cocaine concentrations after the injection of thioperamide and cocaine

Thioperamide enhanced plasma cocaine concentrations over the entire 60-min session (Fig. 4). Two-way ANOVA applied on the cocaine concentrations indicated a significant effect of thioperamide ($F_{1,40}=63.667$, $P<0.001$) and a significant time effect ($F_{3,40}=41.355$, $P<0.001$). The interaction was close to significance ($F_{3,40}=2.755$, $P=0.054$). Similarly, cocaine's $AUC_{0\text{-}60\ min}$ and C_{max} were increased approximately twofold after thioperamide treatment (Table 2).

Experiment 12: effects of thioperamide on cocaine-induced hyperactivity in wild-type and HDC KO mice from the 129/Sv background

Thioperamide (10 mg/kg) slightly increased the locomotor effects of cocaine in wild-type 129/Sv mice (Fig. 5A). The

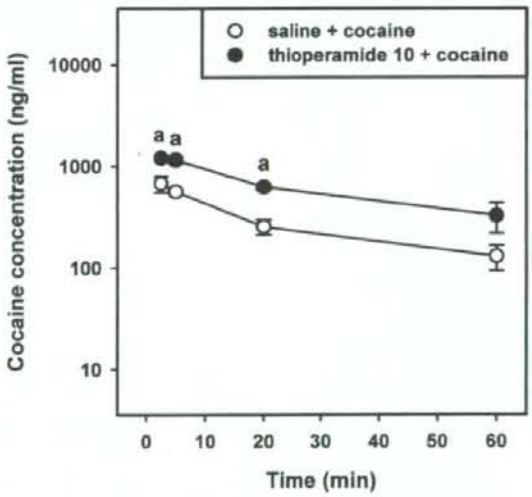

Fig. 4 Plasma cocaine concentrations (ng/ml of plasma) after the injection of thioperamide (10 mg/kg, i.p.) and cocaine (8 mg/kg, i.p.) in C57BL/6J mice. Mice were decapitated 2.5, 5, 20, or 60 min after the cocaine injection. *a* Significantly different from the group injected with cocaine alone at a given time point

Table 2 Pharmacokinetics of cocaine after the administration of thioperamide (10 mg/kg, ip) in C57BL/6J mice

	Saline and cocaine	Thioperamide and cocaine	*t* test
$AUC_{0-60\ min}$ (ng min/ml)	16,227.3±1,632.4	36,859±3,060.5	$t_{10}=5.948$, $P<0.001$
C_{max} (ng/ml)	678.5±124.4	1,204±70.5	$t_{10}=3.674$, $P<0.01$

three-way ANOVA for the whole session revealed a significant thioperamide by cocaine by time-course interaction ($F_{5,160}=3.525$, $P<0.01$), a significant effect of cocaine ($F_{1,32}=23.279$, $P<0.0001$) and a significant effect of thioperamide ($F_{1,32}=4.576$, $P<0.05$). Post-hoc tests indicated that thioperamide potentiated cocaine-induced activity during the fourth and the fifth 10-min intervals of the session (Fig. 5A). The two-way ANOVA for the first 20 min only revealed a significant effect of cocaine ($F_{1,32}=11.433$, $P<0.001$) indicating that wild-type 129/Sv mice were stimulated by cocaine during that time interval (Fig. 5C).

Thioperamide at 10 mg/kg strongly enhanced cocaine-induced locomotion over the whole session in HDC KO mice (Fig. 5D–F). The three-way ANOVA for the entire session revealed a significant effect of cocaine ($F_{1,32}=35.976$, $P<0.001$), thioperamide ($F_{1,32}=30.271$, $P<0.001$) and a significant thioperamide by cocaine interaction ($F_{1,32}=24.517$, $P<0.001$). The two-way ANOVA for the first 20 min revealed a significant effect of cocaine ($F_{1,32}=$

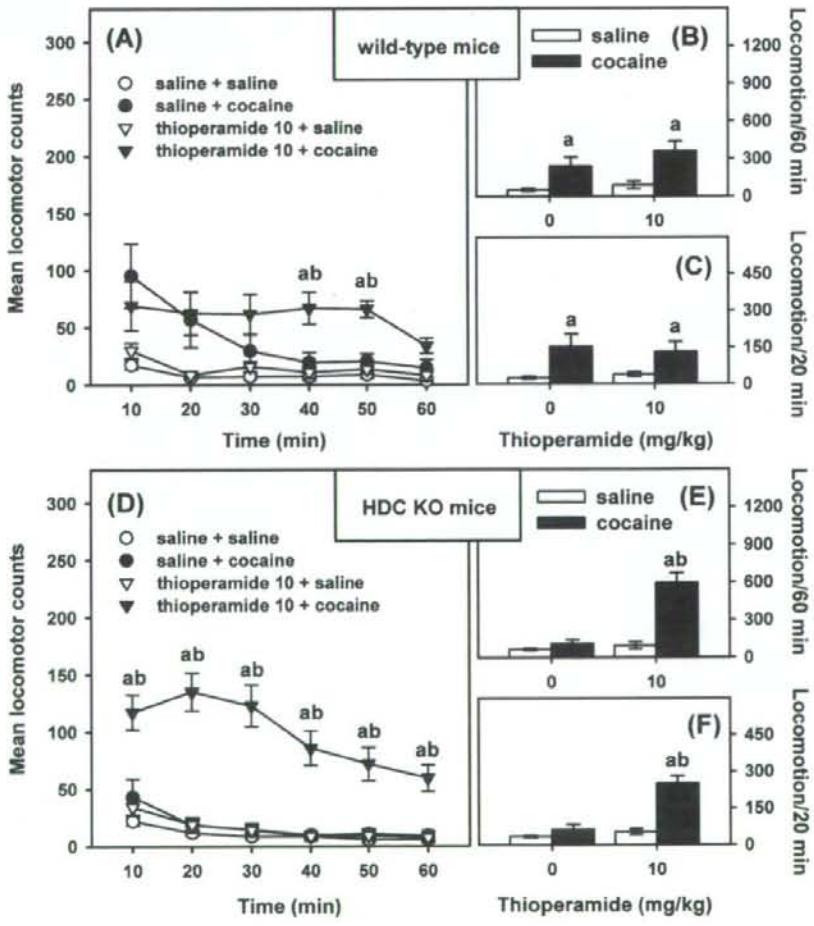

Fig. 5 Effects of thioperamide (10 mg/kg, i.p.) on spontaneous locomotion and on cocaine-induced hyperactivity (8 mg/kg, i.p.) in wild-type (**A**–**C**) and HDC KO mice (**D**–**F**) from the 129/Sv background. Locomotion is expressed as mean (±SEM) number of locomotor counts. **A**, **D** Time course of locomotion in 10-min intervals. **B**, **E** Total activity for the whole session. **C**, **F** Total activity for the first 20 min of the session. *a* Significantly different from the respective group injected without cocaine; *b* significantly different from the respective group injected with cocaine alone

26.878, $P<0.001$), thioperamide ($F_{1,32}=23.240$, $P<0.001$), and a significant thioperamide by cocaine interaction ($F_{1,32}=14.890$, $P<0.001$). In contrast to their wild-type counterparts, HDC KO mice were not significantly stimulated by cocaine alone ($P=0.356$; Fig. 5F).

Discussion

Our results replicate previous findings, which showed that thioperamide potentiates the locomotor effects of cocaine in C57BL/6J mice (Brabant et al. 2005, 2006) and generalizes this effect to low doses of thioperamide (2.5 mg/kg) and to 129/Sv mice. In addition, the H_3 agonist immepip significantly reduces the action of thioperamide on cocaine-induced locomotion. The non-imidazole H_3 inverse agonist A-331440 only slightly affects the locomotor effects of cocaine. Moreover, plasma cocaine concentrations are more elevated in mice treated with thioperamide than in mice that received cocaine alone. Finally, the ability of thioperamide to enhance cocaine-induced hyperactivity is stronger in HDC KO mice than in their wild-type counterparts.

The present research tested thioperamide on the locomotor effects of 8 mg/kg cocaine. This dose produces a moderate elevation of locomotor activity and is at the bottom section of the dose–response curve of cocaine-induced locomotion in C57BL/6J (Fig. 1) or in 129/Sv mice (Brabant et al. 2007b). In C57BL/6J mice, 10 mg/kg thioperamide elevated the locomotor effects of 8 mg/kg cocaine to levels similar to that induced by doses of cocaine between 16 and 24 mg/kg. These results are in agreement with our previous studies showing that thioperamide strongly enhances the locomotor effects of cocaine (Brabant et al. 2005; 2006). However, our data conflict with an earlier report indicating that thioperamide decreases amphetamine-induced locomotion in mice (Clapham and Kilpatrick 1994). In our previous studies, thioperamide was injected 10 min before cocaine, whereas it was administered 30 min before amphetamine in the study from Clapham and Kilpatrick (1994). Since thioperamide strongly increases histamine in the brain 30 min after i.p. administration (Itoh et al. 1991), a possible explanation for these discrepancies could be the different time intervals between thioperamide and psychostimulant injections that were used in these studies. However, the present research found that thioperamide, injected either 5 or 30 min before cocaine, significantly potentiated the locomotor effects of cocaine (experiment 3). The 5-min time interval was found optimal to increase cocaine-induced activity and was adopted throughout this study, except in experiment 12, which investigated the role of histamine in the potentiating effects of thioperamide on cocaine-induced locomotion.

Another explanation that could explain why thioperamide differentially affects the stimulant effects of cocaine and amphetamine is that the ability of thioperamide to potentiate cocaine-induced hyperactivity is not related to the blockade of H_3 receptors. We have found that the selective H_3 agonist immepip, at doses that did not affect the locomotor effects of cocaine, somewhat reduced the ability of thioperamide to potentiate cocaine-induced locomotion. These results suggest that H_3 receptors play a role in the potentiating effects of thioperamide but that H_3 receptor blockade alone is not sufficient to explain the effects of this compound. It is unlikely that immepip could not block all the potentiating effects of thioperamide because its effects were not long enough to cover the 60-min session during which locomotion was recorded. Immepip (10 mg/kg, i.p.) easily crosses the blood brain barrier and induces pharmacological effects for at least 120 min (Lamberty et al. 2003). Moreover, the ability of immepip to produce antinociception lasts at least 90 min after systemic injection and is blocked completely by an i.p. injection of thioperamide (Cannon et al. 2007). Thus, thioperamide likely increases cocaine-induced hyperlocomotion in part through a mechanism that does not involve H_3 receptors.

Experiments 9 and 10 investigated whether A-331440, a non-imidazole H_3 inverse agonist highly selective for H_3 receptors, could produce similar potentiating effects on cocaine-induced hyperactivity to thioperamide in C57BL/6J mice (Hancock et al. 2004). A-331440 did not significantly increase the locomotor effects of cocaine when this compound was tested over different doses (2.5–20 mg/kg) and at different time intervals before the cocaine injection (5 or 60 min). The analysis of the time course of the effects of thioperamide and A-331440 indicates important differences between these two compounds on cocaine-induced hyperactivity. In C57BL/J mice, thioperamide produced the strongest potentiation when injected at 10 mg/kg 5 min before cocaine. Under these conditions, thioperamide elevated the locomotor effects of cocaine by about 392 to 572 locomotor counts over the whole 60-min session (experiments 3, 4, and 7). In contrast, when injected 1 h before cocaine administration, 5 mg/kg A-331440 non-significantly enhanced cocaine-induced locomotion by only 85 locomotor counts over the first 20 min of the session (experiment 10). The calculation of ANOVA-based measures of effect size (η^2) also supports that A-331440 did not elevate cocaine-induced hyperlocomotion to the same extent as thioperamide in the present study. In experiments 3 and 4, the effect sizes of thioperamide on cocaine-induced locomotion were between 0.271 and 0.528 and were more than two times higher than those of A-331440 (which were between 0.024 and 0.116 in experiments 9 and 10). Our results suggest that thioperamide and A-331440 differentially affect cocaine-induced locomotion and confirm the

hypothesis that thioperamide increases the locomotor effects of cocaine in part through a mechanism that does not involve H_3 receptors.

Thioperamide (10 mg/kg) injected 5 min before cocaine increased plasma cocaine concentrations and $AUC_{(0-60\ min)}$ by more than twofold 1 h after cocaine administration. Consequently, pharmacokinetic interactions between thioperamide and cocaine likely contribute to the ability of thioperamide to increase cocaine-induced locomotion. Although the limited number of time points precluded accurate calculation of i.p. clearances (Cl/F), inspection of the semilog plot (Fig. 4) indicates that cocaine's half-life was not significantly altered by thioperamide. Thus, cocaine's bioavailability by the i.p. route appears to be markedly enhanced following thioperamide treatment. Since intraperitoneal administered cocaine is largely taken up by the portal circulation, the likely mechanism is inhibition of hepatic first pass metabolism by thioperamide through the blockade of cytochrome P450 enzymes. Thioperamide is an imidazole-based compound and, like many drugs that bear an imidazole group, strongly inhibits P450 cytochromes (Yang et al. 2002), the primary enzymes involved in the *N*-demethylation of cocaine in mice, rats, and humans (Pellinen et al. 1994, 2000; Poet et al. 1996). It is thus possible that thioperamide increased plasma cocaine concentrations in the present study through the blockade of the cytochrome P450 enzymes that contribute to the elimination of cocaine and that the increased cocaine concentration produced more locomotor stimulation. This interpretation is supported by the lack of significant potentiating effects of A-331440, a non-imidazole based H_3 inverse agonist that does not block cytochrome P450 enzymes (Hancock et al. 2004), on cocaine-induced activity. Moreover, a preliminary study found that clobenpropit, an imidazole-based H_3 inverse agonist that blocks cytochrome P450 enzymes (Yang et al. 2002), increases cocaine-induced locomotion in 129/Sv mice (Brabant et al. 2007a). Our study suggests thus that a pharmacokinetic interaction between thioperamide and cocaine might explain, at least in part, the differential actions of thioperamide on the locomotor effects of cocaine and amphetamine.

In addition to a pharmacokinetic interaction, it is likely that the blockade of H_3 receptors has also contributed to the ability of thioperamide to increase cocaine-induced locomotion in the present study. Indeed, the selective H_3 agonist immepip significantly reduced the ability of 2.5 and 10 mg/kg thioperamide to increase the locomotor effects of cocaine. Experiment 12 investigated whether histamine is involved in the potentiating effects of thioperamide on cocaine-induced hyperlocomotion. In agreement with our previous study (Brabant et al. 2007b), HDC KO mice, unlike their wild-type counterparts from the 129/Sv background, were not significantly stimulated by 8 mg/kg cocaine. However, the activity of these knockout mice was markedly enhanced when thioperamide was injected before cocaine, indicating that an increased release of histamine produced by the blockade of H_3 autoreceptors is not required for the ability of thioperamide to potentiate cocaine-induced hyperactivity. The present study rather suggests that H_3 receptors located on non-histaminergic neurons are involved in the modulation of the locomotor effects of cocaine by thioperamide. GABAergic postsynaptic H_3 receptors have been shown to display high constitutive activity in the striatum (Humbert-Claude et al. 2007). Thioperamide, acting as a H_3 inverse agonist, could thus potentially increase cocaine-induced hyperlocomotion through the blockade of these receptors in mice lacking HDC. This hypothesis is in agreement with the recent results of Ferrada et al. (2008). They found that thioperamide (3 and 10 mg/kg, i.p.) increases the locomotor effects of the selective D_1 agonist SKF38393 in reserpinized mice, very likely, through the blockade of postsynaptic H_3 receptors located in the striatum.

Although the underlying molecular mechanisms of the present data remain speculative, it is possible that postsynaptic H_3 receptors modulate cocaine-induced locomotion through the inhibition of the signaling cascades downstream of ventrostriatal D_1 receptors known to play an essential role in the locomotor effects of cocaine (Borgkvist and Fisone 2007). It has been shown that postsynaptic H_3 and D_1 receptors are co-localized on GABAergic neurons in the striatum (Ryu et al. 1994). Moreover, the activation of H_3 receptors inhibits D_1-receptor-mediated cyclic AMP (cAMP) accumulation in rat striatal slices (Sanchez-Lemus and Arias-Montano 2004). Therefore, Ferrada et al. (2008) proposed that thioperamide increases the locomotor effects of SKF38393 through the blockade of postsynaptic H_3 receptors that normally inhibit the D_1-receptor-mediated adenyl cyclase/cAMP cascade in striatonigral GABAergic neurons. This molecular pathway plays a major function in the locomotor stimulant effects of cocaine (Borgkvist and Fisone 2007). It is thus possible that thioperamide enhanced cocaine-induced locomotion in this study in part through molecular mechanisms similar to that proposed by Ferrada et al. (2008) for SKF38393.

It is important to note that an increase of endogenous histamine produced by H_3 inverse agonists through the blockade of H_3 autoreceptors can potentially activate H_3 receptors present on non-histaminergic neurons. In particular, thioperamide (2–10 mg/kg, i.p.) strongly increases histamine in the brain of mice and rats (Itoh et al. 1991; Taylor et al. 1992). Since histamine's affinity for H_3 receptors is high (Schwartz et al. 1986), it can compete with the ability of thioperamide to fully block postsynaptic H_3 receptors, namely, in the ventral striatum where histaminergic fibers are present (Alves-Rodrigues et al.

1996; Panula et al. 1989). Consequently, the potentiating effects of thioperamide on cocaine-induced hyperactivity resulting from the blockade of postsynaptic H_3 receptors might be reduced by a concomitant histamine release. This could explain why A-331440, when injected 60 min before cocaine, produced only a slight non-significant increase of cocaine-induced locomotion in wild-type C57BL/6J mice in the present study. In agreement with that view, thioperamide's effects on histamine synthesis were mimicked by A-331440 in brain cortical slices (Moreno-Delgado et al. 2006). The same explanation could clarify why thioperamide potentiated cocaine-induced locomotion to higher levels in HDC KO mice than in their wild-type counterparts from the 129Sv strain since endogenous histamine cannot be increased by thioperamide in the absence of HDC (Fig. 5).

It is possible that low doses of thioperamide could potentiate cocaine-induced hyperactivity through the blockade of H_3 receptors without inhibiting cytochrome P450 enzymes. However, the range of doses of thioperamide able to produce such effects are likely to be narrow in C57BL/6J mice. Indeed, immepip was not more effective in reversing the potentiating effects of 2.5 mg/kg thioperamide as compared to 10 mg/kg in the present study. Zhang et al. (2005) found that 3.2 mg/kg thioperamide (i.p.) potentiates risperidone-induced catalepsy in rats through the inhibition of cytochrome P450 activity. Moreover, in an in vitro study, it was suggested that thioperamide binds to P450 cytochromes at nanomolar concentrations (Alves-Rodrigues et al. 1996). It is thus likely that immepip could not completely block the potentiating effects of 2.5 mg/kg thioperamide on cocaine-induced locomotion in our study because this dose was high enough to inhibit cytochrome P450 enzymes. Taken together, these data suggest that the high affinity of thioperamide for cytochrome P450 enzymes is important to consider when interpreting results from studies involving the co-administration of thioperamide and cocaine. In particular, thioperamide (7.5–20 mg/kg, i.p.) increases the rewarding and subjective properties of cocaine, an effect assumed to result from the blockade of striatal H_3 receptors (Mori et al. 2002; Campbell et al. 2005; Brabant et al. 2005). The present data suggest that pharmacokinetic effects might also contribute to the ability of thioperamide to potentiate the rewarding and subjective effects of cocaine. However, the extent to which pharmacokinetic interactions are involved in these effects of thioperamide may depend on different factors, such as the dose of thioperamide and the strain of mouse tested. For example, we found that 10 mg/kg thioperamide injected 30 min before cocaine produced a lower increase of cocaine-induced hyperlocomotion in wild-type 129/Sv mice (Fig. 5A–C) than in C57BL/6J mice (Fig. 2D–F). Our results suggest that 10 mg/kg thioperamide primarily increases cocaine-induced hyperactivity through the inhibition of the cytochrome-P450-mediated pathway, which has been reported to be particularly active in the metabolism of cocaine in C57BL/6J mice (Leibman et al. 1990). Therefore, strain-related differences in the activity of cytochrome-P450-mediated pathways could explain why thioperamide potentiated cocaine-induced locomotion to a higher extent in C57BL/6J mice than in 129Sv mice in this study.

In conclusion, the current results indicate that the ability of thioperamide to potentiate cocaine-induced hyperactivity does not result from an increased release of histamine produced by the blockade of H_3 autoreceptors. Our data suggest that intraperitoneal injections of thioperamide, at least at 10 mg/kg, increase the locomotor effects of cocaine in mice through two independent mechanisms: (1) a pharmacokinetic drug–drug interaction between thioperamide and cocaine produced by the blockade of cytochrome P450 enzymes, and potentially, (2) the blockade of postsynaptic H_3 receptors located in the ventral striatum.

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Cutting Edge

THE JOURNAL OF IMMUNOLOGY

Cutting Edge: Histamine Receptor H4 Activation Positively Regulates In Vivo IL-4 and IFN-γ Production by Invariant NKT Cells[1]

Maria C. Leite-de-Moraes,[2*] Séverine Diem,[*] Marie-Laure Michel,[*] Hiroshi Ohtsu,[†] Robin L. Thurmond,[‡] Elke Schneider,[*] and Michel Dy[*]

Histamine (HA) is a biogenic amine with multiple activities in the immune system. In this study we demonstrate that histamine-free histidine decarboxylase-deficient ($HDC^{-/-}$) mice present a numerical and functional deficit in invariant NK T (iNKT) cells as evidenced by a drastic decrease of IL-4 and IFN-γ production. This deficiency was established both by measuring cytokine levels in the serum and intracellularly among gated iNKT cells. It resulted from the lack of HA, because a single injection of this amine into $HDC^{-/-}$ mice sufficed to restore normal IL-4 and IFN-γ production. HA-induced functional recovery was mediated mainly through the H4 histamine receptor (H4R), as assessed by its abrogation after a single injection of a selective H4R antagonist and the demonstration of a similar iNKT cell deficit in $H4R^{-/-}$ mice. Our findings identify a novel function of HA through its H4R and suggest that it might become instrumental in modulating iNKT cell functions. *The Journal of Immunology*, 2009, 182: 1233–1236.

Histamine (HA)[3] is one of the most versatile biogenic amines with multiple physiological functions in the CNS, the intestinal tract, and inflammatory reactions. More recently, a number of studies have established that besides its most obvious contribution to allergic reactions, HA also exerts more subtle regulatory functions influencing the orientation of the immune response, thus rekindling interest in this field of investigation (1–4). It has been assumed until lately that these immunomodulatory effects were mediated mainly through classical HA receptors of the H1 and H2 subtypes (5, 6). However, this explanation has since been complicated by the identification of organic cation transporter 3, OCT3, as a means through which HA can be taken up by murine basophils and exert a negative feedback on their HA, IL-4, IL-6, and IL-13 production (7), as well as by the identification of an additional HA receptor. HA receptor subtype 4 (H4R) expressed mainly in hematopoietic and immunocompetent cells (8, 9). The most clearly established activities of H4R consist in the recruitment and activation of cells involved in inflammatory responses such as eosinophils, mast cells, neutrophils, conventional T lymphocytes, and dendritic cells (10–13). However, its functional expression in the immunoregulatory invariant NK T (iNKT) cells has not been investigated so far.

iNKT cells constitute a distinctive population of mature T lymphocytes positively selected by the nonpolymorphic MHC class-I-like molecule CD1d. They coexpress a highly restricted TCR repertoire composed of a single invariant Vα14Jα18 chain in mice and a Vα24Jα18 chain in humans, preferentially paired with a limited TCR Vβ-chain repertoire that specifically recognizes glycolipids (14–16). iNKT cells are implicated in the control of several immune responses, most likely because of their capacity to promptly produce several cytokines (14–20) such as IL-4 and IFN-γ. In the present study we demonstrate that HA participates in this functional tuning to ensure optimal IL-4- and IFN-γ production by iNKT cells.

Materials and Methods

Animals

Male C57BL/6J mice (7–9 wk old) were purchased from Janvier. Histidine decarboxylase (HDC)-deficient ($HDC^{-/-}$) and $H4R^{-/-}$ mice, backcrossed 12 and 10 times to C57BL/6J mice, respectively (21, 22), were bred in our own facilities. $HDC^{-/-}$ mice received a histamine-low diet (SAFE Scientific Animal Food and Engineering) to avoid exogenous uptake. Animal experiments were performed according to the French institutional committee.

*Faculté de Médecine René Descartes, Centre National de la Recherche Scientifique Unité Mixte de Recherche 8147, Hôpital Necker, Paris, France; †Tohoku University Graduate School of Engineering, Sendai, Japan; and ‡Johnson & Johnson Pharmaceutical Research and Development, San Diego, CA 92121

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[2] Address correspondence and reprint requests to Dr. Maria C. Leite-de-Moraes, Centre National de la Recherche Scientifique Unité Mixte de Recherche 8147, Faculté de Médecine René Descartes, Paris V, Hôpital Necker, 161 Rue de Sèvres, 75743, Paris, Cedex 15, France. E-mail address: leite.de.moraes@necker.fr

[3] Abbreviations used in this paper: HA, histamine; α-GalCer, α-galactosylceramide; H4R, HA receptor subtype 4; HDC, histidine decarboxylase; iNKT, invariant NK T (cell); MNC, mononuclear cell.

In vivo treatment

Mice received a single i.p. administration of 2 μg of α-galactosylceramide (α-GalCer; Alexis) 90 min before sacrifice. In some experiments, mice were injected i.p. 1 h before α-GalCer administration with a single dose of HA (Sigma-Aldrich) (20 mg/kg) with or without the H4R antagonist JNJ 7777120 (23) (20 mg/kg) administered i.p. 1 h before HA injection.

Cell preparation

Lymphocytes were isolated from the spleen using a homogenizer. Mononuclear cells (MNC) were separated from hepatocytes and cellular debris by way of a 35% isotonic Percoll density gradient (Amersham Biosciences). Liver and spleen MNC were depleted of RBC using red cell lysis buffer (8.3 mg/ml NH_4Cl, 1 mg/ml $KHCO_3$, and 3.72 μg/ml EDTA).

Flow cytometry

Splenocytes and liver MNC were preincubated with mAbs against FcγR (clone 2.4G2 culture supernatant), washed, and incubated with CD1d-α-GalCer tetramer-allophycocyanin or control tetramers, anti-CD4 PerCP-Cy-5.5, anti-TCRβ-FITC, anti-IL-4-PE, anti-IFN-γ-PE, or isotype control (BD Pharmingen) as described (20). In some experiments an anti-H4R (clone Y-19; Santa Cruz Biotechnology) was used according to the manufacturer's instructions. Cells were analyzed on a FACSCanto II (BD Biosciences) flow cytometer using FACSDiva software.

Determination of cytokines

IL-4 and IFN-γ were measured by ELISA as described (20).

Statistical analysis

The nonparametric test t was used to calculate significance levels for all measurements. Values of $p < 0.05$ were considered statistically significant.

Results and Discussion

IL-4 and IFN-γ production by iNKT cells is decreased in HA-free $HDC^{-/-}$ mice

Prompt production of IL-4 and IFN-γ in response to TCR cross-linking constitutes a typical feature of iNKT cells. We measured these cytokines to establish whether exogenous HA participated in their modulation. To this end, we injected wild-type and histamine-free $HDC^{-/-}$ mice (deficient for the HA-forming enzyme HDC) with α-GalCer, a glycolipid widely used as a specific activator of iNKT cells, to determine its capacity to specifically activate and promptly induce these cytokines. We found that both IL-4 and IFN-γ levels generated after a single injection were significantly lower in the serum of $HDC^{-/-}$ mice than in wild-type controls (Fig. 1, *A* and *B*). This decrease could result either from a lower incidence or a functional defect of iNKT cells. Indeed, we found that CD1d/α-GalCer tetramer$^+$ cells were effectively reduced in spleen and liver of $HDC^{-/-}$ mice, both in terms of cell counts and percentage (Fig. 1, *C–E*).

The lower iNKT cell counts in histamine-free mice do not exclude the presence of functional deficiencies in the remaining cells, promoting us to analyze cytokine production in single cells by intracellular staining. It turned out that among gated iNKT cells the percentage that was actually positive for IL-4 and IFN-γ cells after injection of α-GalCer was strikingly reduced in $HDC^{-/-}$ mice compared with controls (Fig. 2, *A* and *B*). These data clearly show that iNKT cells are both numerically and functionally impaired in histamine-deficient mice.

HA injection restores the IL-4- and IFN-γ-producing capacity of iNKT cells

To confirm the implication of HA in the cytokine-producing capacity of iNKT cells, $HDC^{-/-}$ mice were treated with HA 1 h before α-GalCer stimulation. Remarkably, a single injection of HA was sufficient to restore the seric levels of IL-4 and IFN-γ in $HDC^{-/-}$ mice (Fig. 1, *A* and *B*). Even though this treatment did not enhance the percentage or the absolute number of iNKT cells significantly (data not shown), it did increase the proportion of IL-4$^+$ and IFN-γ$^+$ cells among gated iNKT lymphocytes (Fig. 2), consistent with the restored seric cytokine levels. It can therefore be concluded that HA is capable of up-regulating both IL-4 and IFN-γ production by iNKT cells activated in vivo.

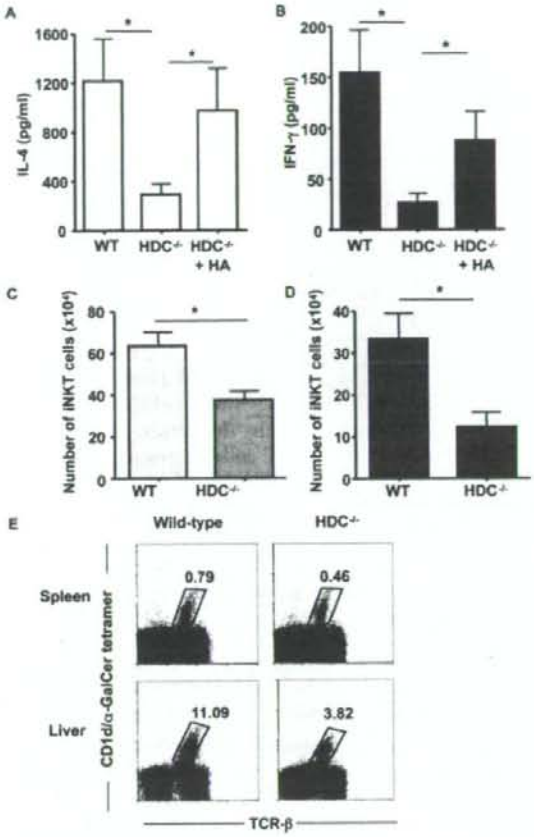

FIGURE 1. Marked decrease in cytokine production by iNKT cells in $HDC^{-/-}$ mice. Wild-type (WT) and $HDC^{-/-}$ mice were injected with α-GalCer. A group of $HDC^{-/-}$ mice were treated with HA 1 h before α-GalCer injection ($HDC^{-/-}$ + HA). Ninety minutes later, IL-4 (*A*) and IFN-γ (*B*) were measured in the serum. No cytokine was detected without α-GalCer stimulation (data not shown). The total number (*C* and *D*) and the percentage (*E*) of iNKT cells were analyzed in both spleen (*C* and *E*) and liver (*D* and *E*) from untreated wild-type or $HDC^{-/-}$ mice. Data represent the mean ± SEM from 8–20 mice (*A* to *D*). *, $p < 0.05$. Representative FACS profiles showing the percentage of CD1d/α-GalCer$^+$TCRβ$^+$ cells (*E*) in wild-type vs $HDC^{-/-}$ mice.

Cytokine production by iNKT cells is impaired in H4 receptor-deficient mice

Knowing that histamine exerts its biological effect through four specific receptors and that the most recently discovered H4 subtype is preferentially expressed in hematopoietic cells, we used mice in which the corresponding gene had been disrupted ($H4R^{-/-}$) to assess their IL-4 and IFN-γ production following

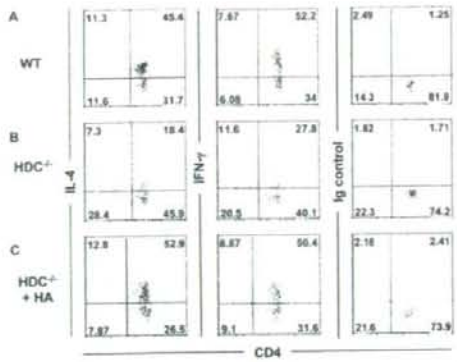

FIGURE 2. Impaired IL-4 and IFN-γ production by in vivo α-GalCer-stimulated iNKT cells from $HDC^{-/-}$ mice Representative FACS profiles showing the percentage of IL-4, IFN-γ, and isotype control (Ig control) positive cells among gated CD1d/α-GalCer tetramer$^+$TCRβ$^+$ iNKT splenocytes from α-GalCer treated wild-type (WT) (*A*) or $HDC^{-/-}$ mice (*B*). In the later group, $HDC^{-/-}$ mice were treated with HA ($HDC^{-/-}$ + HA) before α-GalCer injection (*C*).

α-GalCer injection. We found that $H4R^{-/-}$ mice, which presented no significant modification in the absolute number of iNKT cells ($5.2 \times 10^5 \pm 0.9 \times 10^5$ vs $6.7 \times 10^5 \pm 1.2 \times 10^5$ splenic iNKT cells from wild-type and $H4R^{-/-}$ mice, respectively), generated significantly fewer circulating cytokines than wild-type controls (Fig. 3, *A* and *B*), suggesting that the positive effect of HA on these biological activities was mediated through H4R activation.

In vivo treatment of $HDC^{-/-}$ mice with a H4R antagonist abrogates the restoration of iNKT cell functions in response to HA

To prove that H4Rs were required for the up-regulation of IL-4 and IFN-γ production by iNKT cells, we blocked their binding sites with the highly selective H4 antagonist JNJ7777120 injected 1 h before HA into $HDC^{-/-}$ mice. In this case HA failed to restore a normal IL-4 and IFN-γ production, as shown in Fig. 3, *C* and *D*. In further support of this result, we show that iNKT cells express H4R (Fig. 3*E*), leading us to conclude that HA modulates iNKT cell functions through this receptor subtype.

The contribution of iNKT cells to immune responses is complex because of their capacity to produce both IFN-γ and IL-4, thereby supporting Th1 or Th2 responses, respectively. We and others have reported that iNKT cells can aggravate asthma through their Th2 cytokine profile (18, 24). Similarly, HA plays a major role in atopic diseases, namely in allergic asthma, because its release in the airways is one of the typical features of this pathology that triggers a cascade of events, including airway constriction, mucus secretion, vascular leak, and recruitment of immune cells. Our present data suggest an additional means for HA to enhance the severity of asthma by promoting optimal IL-4 production by iNKT cells. Consistent with this assumption, it has recently been reported that asthmatic mice treated with the JNJ7777120 antagonist used herein develop less airway inflammation than untreated controls (11).

Taken together, our data reveal a new role of histamine through H4R activation in the functional modulation of the immunoregulatory iNKT cell population and provide additional evidence for the complex influence of the microenvironment on iNKT cell functions.

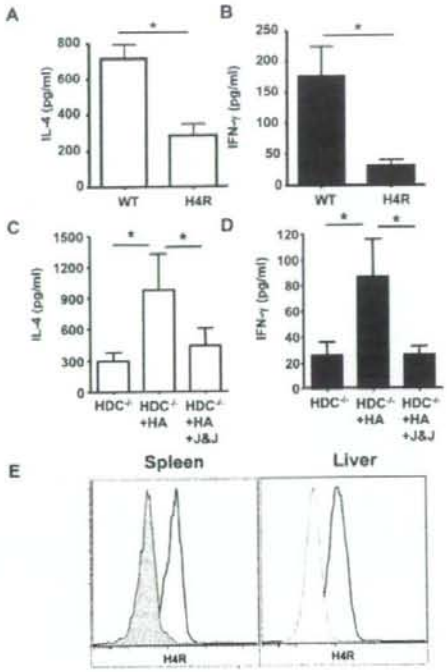

FIGURE 3. HA can modulate iNKT cell functions through H4R. *A* and *B*, Wild-type (WT) and $H4R^{-/-}$ mice were injected with α-GalCer. Ninety minutes later, IL-4 (*A*) and IFN-γ (*B*) were measured in the serum. *C* and *D*, $HDC^{-/-}$ mice were treated or not with the selective H4R antagonist JNJ7777120 (J&J), followed by HA and then α-GalCer injection as described in *Materials and Methods*. Ninety minutes after the last injection, IL-4 (*C*) and IFN-γ (*D*) were measured in the serum. Data represent the mean ± SEM from 6–10 mice: *, $p < 0.05$. *E*, Representative FACS profiles showing the expression of H4R (empty histogram) compared with control (filled histogram) among gated CD1d/α-GalCer$^+$TCRβ$^+$ splenocytes (*left panel*) or liver MNC (*right panel*) recovered from wild-type mice.

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Disclosures

The authors have no financial conflict of interest.

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Dietary Histidine Ameliorates Murine Colitis by Inhibition of Proinflammatory Cytokine Production From Macrophages

AYATOSHI ANDOU,*,‡ TADAKAZU HISAMATSU,* SUSUMU OKAMOTO,* HIROSHI CHINEN,* NOBUHIKO KAMADA,* TAKU KOBAYASHI,* MASAKI HASHIMOTO,† TOMOHISA OKUTSU,† KAZUTAKA SHIMBO,§ TOMOKO TAKEDA,† HIDEKI MATSUMOTO,‡ ATSUSHI SATO,‖ HIROSHI OHTSU,‖ MANABU SUZUKI,‡ and TOSHIFUMI HIBI*

*Department of Internal Medicine, School of Medicine, Keio University, Tokyo; ‡Pharmaceutical Research Laboratories, Pharmaceutical Company, Ajinomoto Co, Inc, Kawasaki; §Basic Analytical Chemistry Group, Institute of Life Sciences, Ajinomoto Co, Inc, Kawasaki; and ‖Applied Quantum Medical Engineering, Graduate School of Engineering, Tohoku University, Sendai, Japan

Background & Aims: **Elemental diet (ED) is effective for human Crohn's disease (CD). Although some of this effectiveness may be due to its low antigenic load and low fat content, the mechanisms remain unclear. We sought to assess the role of histidine, one of the constituent amino acids of ED, in controlling colitis.** ***Methods:*** **The interleukin (IL)-10-deficient (IL-10$^{-/-}$) cell transfer model of colitis was used. SCID mice with colitis induced by transfer of IL-10$^{-/-}$ cells were maintained on experimented diets containing either single amino acids or a mixture. The severity of colitis was assessed by wet colon weight. Colonic tumor necrosis factor (TNF)-α messenger RNA (mRNA) expression was detected by quantitative reverse-transcription polymerase chain reaction. Mouse peritoneal macrophages were stimulated by lipopolysaccharides (LPS), with or without amino acids. The concentration of cytokines in the supernatant was determined by enzyme-linked immunosorbent assay. Inhibitor of nuclear factor (NF)-κB-α and nuclear p65 were confirmed by immunoblotting.** ***Results:*** **In the IL-10$^{-/-}$ transfer model, dietary histidine, but not alanine, reduced histologic damage and colon weight and TNF-α mRNA expression. Histidine inhibited LPS-induced TNF-α and IL-6 production by mouse macrophages in a concentration-dependent manner, whereas alanine or histidine-related metabolites had no such effect. Histidine inhibited LPS-induced NF-κB in macrophages.** ***Conclusions:*** **These results showed that histidine could be a novel therapeutic agent for CD by inhibition of NF-κB activation, following down-regulation of proinflammatory cytokine production by macrophages. Thus, our studies provided new insights into the roles of amino acid metabolism in the pathophysiology of CD and for therapeutic strategies.**

Crohn's disease (CD), one of the 2 major forms of inflammatory bowel disease (IBD), is a chronic inflammatory disorder of unknown etiology that primarily affects the gastrointestinal tract.[1,2] Although CD can affect the entire gastrointestinal tract, the small intestine and colon are most commonly involved. Although after years of intensive research the etiology of CD remains unknown, significant progress in recent years has provided new insights. It has been considered that CD is a multifactorial disease composed of genetic backgrounds, environmental factors, and immunologic response. Recent advances in molecular biology and human genomic analysis have demonstrated several IBD susceptibility genes. Several IBD susceptibility genes have recently been identified in Western populations and, except for TNFSF15, appear to contribute to IBD risk in Asian patients.[3–7] Potentially important environmental factors include smoking, diet, and intestinal flora.[7–9] Although CD was rare in Asian countries including Japan, the number of patients with CD in this region has increased during the past decade. One of the reasons for the change to a more Western pattern of intestinal disorders is thought to be due to a more Westernized diet. CD may develop as a dysregulated mucosal immune response to antigens derived from diet or intestinal flora.

Enteral nutrition therapy improves not only nutritional status but also clinical symptoms and morphologic findings of CD and has been used as the first-line therapy for pediatric CD patients.[10–12] In Japan, enteral nutrition, especially elementary diet (ED), has now also become the first line of therapy for adult patients with CD.[13] ED is a low-fat diet and includes amino acids. Some clinical trials have shown ED to be as effective as steroids in achieving short-term remission.[14] Although the mechanisms of the actions of ED remain uncertain, some possibilities have been indicated: (1) a low-antige-

Abbreviations used in this paper: CD, Crohn's disease; ED, elemental diet; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDC, histidine decarboxylase; KC, keratinocyte chemoattractant; LPMCs, lamina propria mononuclear cells; LPS, lipopolysaccharide; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; MLN, mesenteric lymph node; PBMCs, peripheral blood mononuclear cells; TG-PECs, thioglycolate-induced peritoneal exudate cells; TIMP, tissue inhibitors of matrix metalloproteinase.

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nicity diet that reduces the mucosal immune reaction; (2) a low-fat diet is less proinflammatory; and (3) ED alters the enteric flora population. Recently, it has been reported that ED reduces the mucosal production of proinflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-6 in CD.[15] These cytokines play an important role in the inflammatory process in CD. We hypothesize that ED may have mechanisms to suppress intestinal inflammation directly. In this respect, we focused on the effect of amino acids that are the main component of ED. Some amino acids have recently been reported to contribute to the modulation of gut inflammation in a colitis model.[16,17] Here, we demonstrate the efficacy of dietary histidine uptake in an IL-10$^{-/-}$ transfer model of colitis as a T helper cell (Th)-1 disease model that resembles CD.[18] The mechanism of action of histidine was to inhibit nuclear factor (NF)-κB activation in macrophages and suppressed production of proinflammatory cytokines such as TNF-α and IL-6. The anti-inflammatory effects of amino acids suggest a new mechanism of action of ED therapy and, most importantly, the potential of histidine as a novel therapeutic reagent for CD.

Materials and Methods

Reagents

Amino acids used were as follows: L-histidine, carnosine (Sigma-Aldrich, St. Louis, MO) and D-histidine (Wako, Japan). Lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 was also purchased from Sigma-Aldrich. Polyclonal antibodies against inhibitor of NF-κB (IκB)-α and p65 were purchased from Cell Signaling technology (Danvers, MA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively.

Animals

IL-10$^{-/-}$ mice from a C57BL/6 background were purchased from Jackson Laboratory (Bar Harbor, ME). Mice backcrossed for 10 generations with BALB/c mice were used in this study. Wild-type BALB/c mice were obtained from CRJ (Tokyo, Japan), and CB-17/Icr SCID mice were obtained from Japan Clea (Tokyo, Japan). All mice were housed and bred under specific pathogen-free conditions at Pharmaceutical Research Laboratories, Ajinomoto, Japan. The Pharmaceutical Research Laboratories Care and Utilization Committee approved all procedures. In our study, we used 10-week-old male histidine decarboxylase (HDC) gene knockout (HDC$^{-/-}$) mice backcrossed for 6 generations with C57BL/6 mice, described in detail previously,[19-21] and age-/gender-matched C57BL/6N mice (CRJ) as controls. Mice used for this study were generated and bred as homozygous HDC$^{-/-}$ mice at Tohoku University (Sendai, Japan).

Induction of IL-10$^{-/-}$ Cell Transfer Colitis

Colitis was induced in female SCID mice by the adoptive transfer of spleen and mesenteric lymph node cells from diseased IL-10$^{-/-}$ mice, as described previously.[18] Briefly, spleen and mesenteric lymph nodes were removed aseptically from IL-10$^{-/-}$ mice that exhibited diarrhea and weight loss. After single-cell separation, erythrocytes were removed by hypotonic lysis. SCID mice, 8-12 weeks of age were injected intraperitoneally with 200 μL PBS containing 1.0×10^7 IL-10$^{-/-}$ cells. Mice were killed for pathologic evaluation after 3 weeks of treatment.

Assessment of Mouse Models of Chronic Colitis

Experimental diets were fed from day 0 to mice with experimental colitis induced by transfer of IL-10$^{-/-}$ cells into SCID mice (n = 5-8). Mice were killed for assessment of inflammation at 3 weeks after cell transfer. Colitis severity was assessed according to wet colon weight. The transverse colons were excised and used for histologic analysis. Colonic tissue samples were fixed in 10% phosphate-buffered formalin. Paraffin-embedded sections were stained with H&E for light microscopic examination. For immunohistochemistry, tissue samples were snap frozen in OTC medium (Sakura Finetek, Torrance, CA). The primary antibody against F4/80 (eBioscience, San Diego, CA) was detected with FITC-labeled anti-rat IgG antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Cell nuclei in the tissues were counterstained with hematoxylin. Part of the tissue was immediately frozen in liquid nitrogen for subsequent extraction of RNA. The powder form of Charles River Formula (CRF)-1 (Oriental Yeast, Tokyo, Japan) was used as the standard chow. Elental (Ajinomoto, Tokyo, Japan) was used as the ED. A summary of the composition of Elental is shown in Table 1. CRF-1 containing a mixture of the amino acids found in Elental (EDAAs) or single amino acids was used as the experimental diet. The mice were allowed to access the diet and drinking water ad libitum before the study was initiated. After the study was initiated, mice were randomly assigned to receive either the standard chow or the experimental diet in isocaloric amounts.

Collection of Supernatant From Colon Explant Culture

For ex vivo studies, 3-mm punch biopsy specimens were prepared from inflamed colon in IL-10$^{-/-}$ mice. Five biopsy specimens were cultured for 6 hours in 0.5 mL Dulbecco's modified Eagle medium (DMEM) (Sigma-Aldrich) containing 10% (vol/vol) fetal bovine serum (FBS), lipopolysaccharide (LPS) (1 μg/mL), and amino acids. Supernatants were collected and assessed for cytokine secretion by enzyme-linked immunosorbent assay (ELISA).