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Disruption of opioid-induced placebo responses by activation of cholecystokinin type-2 receptors

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Abstract
Rationale Placebos are known to induce analgesia through the activation of μ-opioid receptors in some circumstances, such as after morphine pre-conditioning, an effect that is blocked by opioid antagonists.
Objectives On the basis of the anti-opioid action of cholecystokinin, here we tested whether the activation of the cholecystokinin type-2 receptors abolishes opioid-induced placebo responses.
Methods The activation of the cholecystokinin type-2 receptors was performed by means of the agonist pentagastrin, and placebo responses were obtained after morphine pre-conditioning in an experimental human model of pain (tourniquet technique).
Results Opioid-induced placebo responses were completely disrupted by pentagastrin administration. In addition, a high correlation between the response to morphine and the response to placebo was found, and this correlation was completely abolished by pentagastrin.
Conclusion These results show that the cholecystokinin-2 receptor agonist, pentagastrin, has the same effect as the μ-opioid receptor antagonist, naloxone, on placebo analgesia induced by morphine pre-conditioning, which suggests that the balance between cholecystokinergic and opioidergic systems is crucial in placebo responsiveness in pain. These findings also suggest that cholecystokinin type-2 receptor hyperactivity might be present in placebo non-responders.

Keywords Placebo · Pain · Cholecystokinin · Pentagastrin · Opioids · Morphine

Introduction

In recent times the placebo effect has been analyzed with sophisticated neurobiological tools that have uncovered specific mechanisms at the biochemical, cellular, and anatomical level (Benedetti 2008; Enck et al. 2008; Finniss et al. 2010; Price et al. 2008). Most of our knowledge about these mechanisms comes from the field of pain, where placebos have been found to activate endogenous opioids (Amanzio and Benedetti 1999; Benedetti et al. 2007; Wager et al. 2007; Zubieta et al. 2005) and pain-modulating networks (Eippert et al. 2009a, b; Petrovic et al. 2002; Wager et al. 2004). In particular, the pharmacological experimental approach has shown the opposite action of endogenous opioids on the one hand and cholecystokinin (CCK) on the other. In fact, whereas opioid antagonists block placebo analgesic responses (Amanzio and Benedetti 1999; Benedetti et al. 2007; Eippert et al. 2009a), CCK antagonists have been found both to enhance placebo analgesia (Benedetti et al. 1995) and to block nocebo (anxiety-induced) hyperalgesia both in humans (Benedetti et al. 1997, 2006) and in animals (Andre et al. 2005), thus

emphasizing the pivotal role of CCK in the placebo/nocebo phenomenon.

Whereas in previous studies, on placebo analgesia and nocebo hyperalgesia, CCK was antagonized by means of antagonists, such as the non-specific CCK-1/CCK-2 receptor antagonist, proglumide, in humans (Benedetti et al. 1997, 2006) and the specific CCK-2 receptor antagonist, CI-988, in rodents (Andre et al. 2005), in the present study we enhanced CCK-2 receptor activity by means of pentagastrin. This CCK-2 receptor agonist has been shown to modulate emotional processing in a way that is opposite to μ-opioid agonists such as remifentanil (Gospic et al. 2008), thus suggesting that opioids and CCK may affect complex cognitive/emotional functions.

The pharmacological approach with opioid antagonists, such as naloxone (Amanzio and Benedetti 1999; Benedetti et al. 2007; Eippert et al. 2009a; Levine et al. 1978), and with CCK antagonists, such as proglumide (Benedetti et al. 1995, 1997, 2006), has been crucial over the past years to understand the neurobiology of placebo analgesia. Therefore, in the present study we used a similar approach with a CCK agonist, in order to investigate how CCK hyperactivity affects the placebo analgesic response. To do this, we used a model of placebo response after morphine pre-conditioning, which is known to be mediated by endogenous opioids both in humans (Amanzio and Benedetti 1999; Benedetti et al. 2007) and in rodents (Guo et al. 2010).

Materials and methods

Subjects

The subjects were healthy males and females who agreed to participate in one of the experimental groups after they signed an informed consent form in which the details of the experiment, including the drugs to be administered, were explained. In particular, the subjects were told that either morphine or pentagastrin would be administered at a given time, depending on the experimental group. All the experiments were performed according to the rules of the ethics committee and to the Declaration of Helsinki. We randomly assigned ten subjects to the natural history group (group NH; males/females=5/5, mean age=23±2.2 years, mean weight=64.4±3.1 kg), ten to the hidden pentagastrin group (group HP; males/females=6/4, mean age=23.8±2.8 years, mean weight=69.1±5.3 kg), ten to the placebo group (group PLA; males/females=4/6, mean age=22.8±4.7 years, mean weight=66.7±4.9 kg), and ten to the placebo+pentagastrin group (PLA+PE; males/females=5/5, mean age=23.4±3.9 years, mean weight=67±5.2 kg).

One week before the beginning of the experiments, the subjects underwent a clinical examination, including an electrocardiogram, in order to ascertain their physical conditions and to rule out main diseases. All the subjects were informed that they had to abstain from consuming coffee, tea, and caffeine-containing drinks for 48 h before each session, as well as alcohol and any medication.

Drugs and double-blind procedure

Intravenous morphine was given to groups PLA and PLA+PE during the conditioning phase at a dose of 0.14 mg/kg. It was dissolved in sterile solution of NaCl 0.9%, and the infusion rate was 0.1 ml/s. Intravenous pentagastrin, a CCK type-2 receptor agonist, was given to groups HP and PLA+PE at a dose of 0.1 μg/kg. Drugs were administered 15 min before the induction of pain. Pentagastrin was given according to a randomized double-blind design in group PLA+PE in which neither the subject nor the experimenter knew what drug was being administered. To do this, either the active drug or saline solution was given. To avoid a large number of subjects, two or three additional subjects per group received an intravenous injection of saline in place of the active drug. These subjects were not included in the study because they were used only to allow the double-blind design, as already described by Benedetti et al. (2006, 2007). By contrast, pentagastrin was administered unbeknownst to the subject in group HP.

Experimental pain induction

Pain was induced experimentally by means of the submaximal effort tourniquet technique, according to the procedures described by Amanzio and Benedetti (1999). The subject reclined on a bed, his or her non-dominant forearm was extended vertically, and venous blood was drained by means of an Esmarch bandage. A sphygmomanometer was placed around the upper arm and inflated to a pressure of 300 mmHg. The Esmarch bandage was maintained around the forearm, which was lowered on the subject's side. After this, the subject started squeezing a hand spring exerciser 12 times while his or her arm rested on the bed. Each squeeze was timed to last for 2 s, followed by a 2-s rest. The force necessary to bring the handles together was 7.2 kg. This type of ischemic pain increases over time very quickly, and the pain becomes unbearable after about 14 min. All the subjects were told that they had to tolerate the tourniquet test as long as possible. In order to make the subjects tolerate the pain as long as possible, the tolerance times were taken with steps of 30 s (15, 15.5, 16, 16.5, 17, 17.5 min…), and the subjects were told that they had to complete a full step in order to increase their scores. In other words, if a subject resisted at 16 min and 29 s, his tolerance time was 16, whereas if he resisted at 16 min and 31 s, his tolerance time was 16.5 (Benedetti et al. 2007).

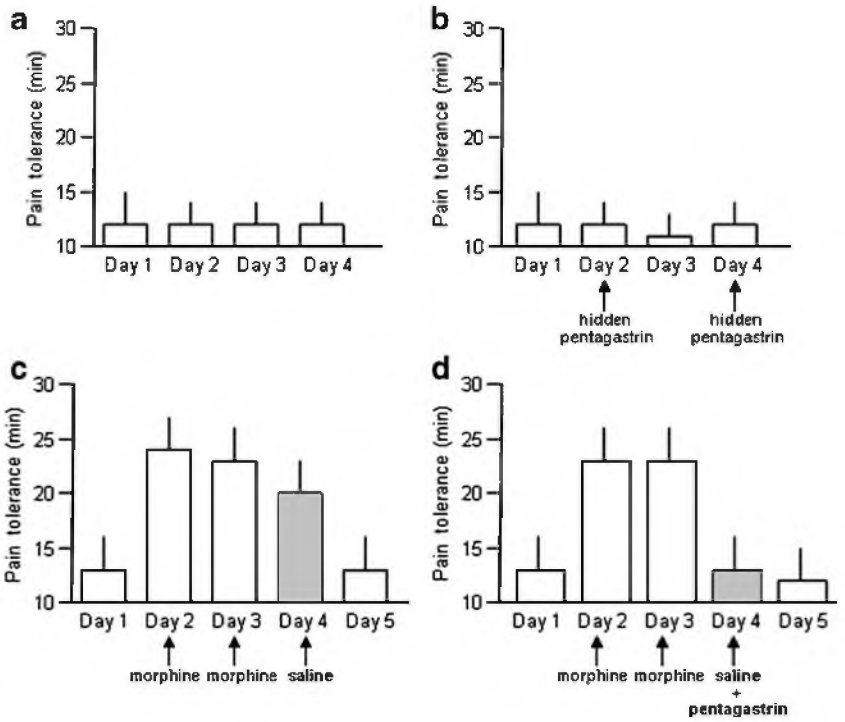

Fig. 1 Results in the different experimental groups. **a** Group NH showed no variations of pain tolerances over a period of four non-consecutive days, and this represents the natural history of pain. **b** Pain tolerances after a hidden injection of pentagastrin in group HP did not differ from no treatment on days 1 and 3, which indicates that this kind of pain is not affected by pentagastrin. **c** In group PLA, after morphine pre-conditioning, saline (placebo) induced a placebo analgesic response that mimicked the effect of morphine. **d** In group PLA+PE, adding pentagastrin to the placebo on day 4 abolished the placebo analgesic effect completely

Experimental procedure

The natural history group (n=10) underwent the tourniquet test and pain tolerance assessment for four non-consecutive days. The inter-day interval was 3–4 days. This group represented the no treatment, or natural history, group and was used to assess the natural course of this kind of pain over the testing period.

The hidden pentagastrin group (n=10) underwent the same procedure, but pentagastrin was injected on days 2 and 4 unbeknownst to the subjects. To do this, saline solution was dripping continuously through the intravenous line, and pentagastrin was added unbeknownst to the subjects. This group was used to see whether pentagastrin per se affected this type of pain.

The placebo group (n=10) was tested over a period of five non-consecutive days. In this case also, the inter-day interval was 3–4 days. On day 1 no treatment was carried out (control), whereas on day 2 and 3 morphine was given as a conditioning drug. On day 4 a placebo (intravenous saline solution) was administered along with verbal suggestions that it was the same morphine with the previous days. Day 5 was the same as day 1 and used as a control. This group was used to elicit a placebo analgesic response after morphine pre-conditioning, according to the procedure used by Amanzio and Benedetti (1999) and Benedetti et al. (2007).

The placebo+pentagastrin (PLA+PE) group (n=10) underwent the very same procedure as the PLA group, but pentagastrin was added to the placebo on day 4 unbeknownst to the subjects. This group was used to see the effects of pentagastrin on the placebo analgesic response.

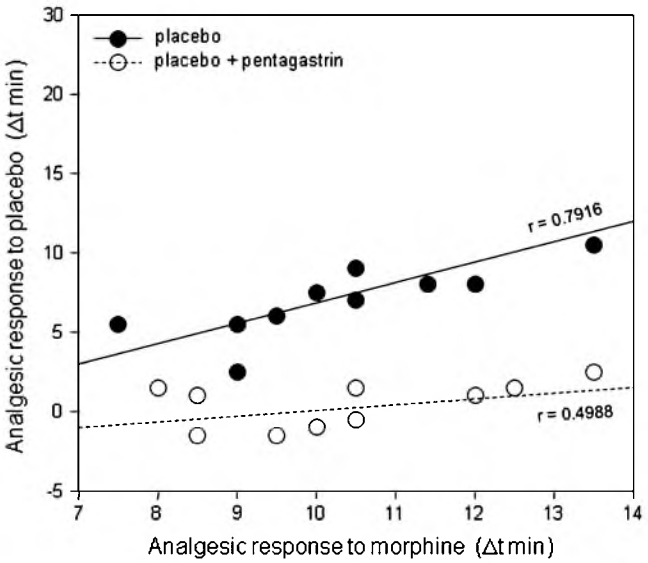

Fig. 2 Relationship between the analgesic response to morphine on day 3 and the analgesic response to placebo on day 4. Each *circle* represents the response of a single subject. The responses are expressed as Δt, i.e., the difference of pain tolerance between days 3 and 1 for morphine and between days 4 and 1 for saline (placebo). The correlation between morphine and placebo is completely disrupted by pentagastrin

Table 1 Group NH (natural history): tolerance times (min) for each subject in each group and statistical analysis within groups

Subject	Day 1	Day 2	Day 3	Day 4
1	10	8	9.5	12
2	10.5	12.5	14	9
3	8	7	9	8.5
4	14	13	10	11.5
5	17.5	14.5	16	17
6	9	10.5	9.5	13.5
7	9	9	10.5	10
8	16	17	14.5	15
9	15.5	14	13.5	16
10	11	12	11	11.5
Mean±SD	12.05±3.4	11.75±3.13	11.75±2.5	12.4±2.9

Repeated measures ANOVA across the 4 days: $F(3,27)=0.438$, $p=0.728$

Statistical analysis

Statistical analysis was performed by means of ANOVA for repeated measures, followed by the post hoc Student–Newman–Keuls test for multiple comparisons and Dunnett test for comparisons between a control group and different experimental groups. In addition, correlations were performed by using linear regression analysis. Comparisons between regression lines were performed by means of the global coincidence test and a slope comparison t test. Data are presented as mean±standard deviation (SD), and the level of significance is $p<0.05$.

Results

The NH group showed no variation in pain tolerance when the tourniquet test was repeated for four non-consecutive days ($F(3,27)=0.438$, $p=0.728$), indicating that it produced pain tolerances that remained constant for several days (Fig. 1a). In addition, the hidden injections of pentagastrin in group HP did not produce any significant variation in pain tolerance compared to days 1 and 3 ($F(3,27)=0.220$, $p=0.881$), which indicates that this kind of experimental pain is not affected by pentagastrin (Fig. 1b).

In group PLA, when morphine was administered on days 2 and 3, a significant increase in pain tolerance was found, indicating its powerful analgesic effect ($F(4,36)=165.086$, $p<0.0001$). A saline injection (placebo) on day 4, which the subjects believed to be morphine, mimicked the morphine responses of the previous days and was significantly different from the control condition of day 1 (post hoc Newman–Keuls $q(36)=16.797$, $p<0.001$), whereas pain tolerance returned to baseline on day 5 (Fig. 1c). If the same procedure was performed but pentagastrin was added to the saline injection on day 4 (group PLA+PE), no morphine-mimicking placebo response could be observed, and day 4 differed neither from day 1 nor from day 5 (Fig. 1d).

A linear regression analysis with the data from single subjects showed a high correlation between the response to morphine on day 3 and the response to saline (placebo) on day 4 ($r=0.7916$, $t(8)=3.664$, $p<0.007$), according to the rule "the larger the morphine responses, the larger the placebo responses" (Fig. 2). In this case, the analgesic response to morphine was expressed as Δt, i.e., the difference between pain tolerance on days 3 and 1. Likewise, the analgesic response to saline (placebo) was expressed as the difference of pain tolerance between days 4 and 1. Pentagastrin disrupted this correlation completely ($r=0.4988$, $t(8)=1.628$, $p=0.142$). A global coincidence test showed a significant difference between the two regression lines ($F(2,16)=57.312$, $p<0.001$).

By looking at the raw data of Tables 1, 2, 3, and 4, it is worth noting that pentagastrin abolished the placebo analgesic responses in all subjects. In fact, in group PLA+PE, none of the subjects showed substantial increases of pain

Table 2 Group HP (hidden pentagastrin): tolerance times (min) for each subject in each group and statistical analysis within groups

Subject	Day 1	Pentagastrin day 2	Day 3	Pentagastrin day 4
1	9	9	9.5	10
2	14.5	14.5	10	15
3	12	12.5	10	11
4	8.5	10	9.5	9
5	10	12	10.5	10.5
6	18	15.5	16	16.5
7	13	13.5	13	13
8	8	8	9.5	9
9	9.5	10	9	9.5
10	15	14	15.5	15
Mean±SD	11.75±3.32	11.9±2.5	11.65±2.7	11.85±2.8

Repeated measures ANOVA across the 4 days: $F(3,27)=0.220$, $p=0.881$

Table 3 Group PLA (placebo): tolerance times (min) for each subject in each group and statistical analysis within groups

Subject	Day 1	Morphine day 2	Morphine day 3	Placebo day 4	Day 5
1	15.5	25.5	24.5	18	14
2	9	19	21	17	10.5
3	11	24	24.5	21.5	13.5
4	14	25	25.5	22	15
5	15.5	24.5	25.5	23	16
6	10.5	22.5	19.5	16	9
7	8.5	20	19	17.5	9.5
8	17	26	26.5	23	16
9	13.5	24.5	21	19	11
10	14.5	27	25	21.5	16
Mean±SD	12.9±2.9	23.8±2.5	23.2±2.8	19.85±2.6	13.05±2.8

Repeated measures ANOVA across the 5 days: $F(4,36)=165.086$, $p<0.0001$

Student–Newman–Keuls: day 4 vs 1: $q(36)=16.797$, $p<0.001$; day 4 vs 2: $q(36)=9.547$, $p<0.01$; day 4 vs 3: $q(36)=8.097$, $p<0.01$; day 4 vs 5: $q(36)=16.435$, $p<0.001$

tolerance on day 4 compared to the control conditions of days 1 and 5. By contrast, in group PLA, all the subjects showed robust pain tolerance increases on day 4.

Discussion

Following previous studies that showed the involvement of endogenous opioids in placebo analgesia (Amanzio and Benedetti 1999; Eippert et al. 2009a; Levine et al. 1978; Wager et al. 2007; Zubieta et al. 2005) and the anti-opioid action of CCK in pain and in complex cognitive/emotional processes (Benedetti et al. 1995, 1997, 2006; Gospic et al. 2008; Hebb et al. 2005), in the present study we tested how the activation of CCK receptors may affect placebo responsiveness. On the basis of the opposite role of endogenous opioids and CCK, we postulated that CCK hyperactivity might result in stronger antagonism against placebo-activated opioids and thus in decreased placebo responsiveness. Indeed, we found that activation of CCK type-2 receptors by means of pentagastrin abolished placebo analgesia completely.

In order to evoke opioid-mediated placebo responses, we used the model of morphine pre-conditioning, whereby pharmacological pre-conditioning with an opioid drug is known to elicit placebo responses that are naloxone-reversible (Amanzio and Benedetti 1999; Benedetti et al. 2007). This effect of morphine pre-conditioning on placebo responses has recently been confirmed in an animal model, whereby morphine pre-conditioned mice showed naloxone-reversible placebo responses whereas non-opioid pre-conditioned mice did not (Guo et al. 2010). Therefore, this represents a nice model of opioid-mediated placebo responses that is amenable to experimental manipulation.

The fact that pentagastrin abolished the placebo analgesic responses in all subjects, as shown in Tables 1, 2, 3, and 4 and Fig. 2, suggests that the hyperactivity of CCK type-2 receptors antagonized the placebo-induced activation of the endogenous opioid systems. In other words, a change in the balance between opioids and CCK, which seems to be

Table 4 Group PLA+PE (placebo+pentagastrin): tolerance times (min) for each subject in each group and statistical analysis within groups

Subject	Day 1	Morphine day 2	Morphine day 3	Placebo+pentagastrin day 4	Day 5
1	11	23	23.5	12.5	12
2	18.5	29	27	16	16.5
3	8.5	19	18.5	7.5	9
4	10	21	23.5	12.5	7.5
5	14	23	24.5	13.5	12
6	13	22.5	23.5	14.5	12
7	9	24.5	21	10	9.5
8	16.5	22	26	15	14
9	16	24	24.5	17	14
10	15	25.5	23	16.5	15.5
Mean±SD	13.15±3.4	23.3±2.7	23.5±2.4	13.5±3	12.2±2.9

Repeated measures ANOVA across the 5 days: $F(4,36)=158.829$, $p<0.0001$

Student–Newman–Keuls: day 4 vs 1: NS; day 4 vs 2: $q(36)=21.562$, $p<0.001$; day 4 vs 3: $q(36)=21.890$, $p<0.001$; day 4 vs 5: NS

crucial in a number of conditions (Benedetti 2008; Enck et al. 2008), may influence placebo analgesia in opposite directions, depending on the activity of these two neurotransmitters. When CCK activity outweighs opioid activity, placebo analgesia is reduced, while the opposite situation leads to increased placebo response.

It is important to note that the hidden administration of pentagastrin in group HP did not have any effect on this type of experimental pain. This is a crucial point in an experimental design like this, as previously emphasized and assessed for naloxone. In fact, in different studies (e.g., Amanzio and Benedetti 1999), naloxone has been tested regarding its effects on experimental ischemic arm pain. Importantly, neither pentagastrin in the present study nor naloxone in other studies (Amanzio and Benedetti 1999) affects this kind of pain, which indicates that the observed pharmacological effects are related to the modulation of placebo analgesia.

It should be noted that one possible limitation of the present study is represented by the fact that we did not test the effects of pentagastrin on those placebo responses that are not mediated by endogenous opioids. It will be interesting to see in future research whether the same effects occur in experimental models whereby placebo analgesia is not reversed by naloxone. It is also worth noting that we obtained these effects in a model of morphine pre-conditioning, thus generalization to other situations cannot be made. Future experiments need to address this point further, in order to understand what happens without a pharmacological conditioning procedure. In other words, it will be interesting to see whether pentagastrin has the same effects on expectation-induced placebo analgesia, with no previous opioid conditioning.

Today we know that at least three neurotransmitters take part in pain modulation by placebos, that is, endogenous opioids (Amanzio and Benedetti 1999; Wager et al. 2007; Zubieta et al. 2005), CCK (Benedetti et al. 1995, 1997, 2006), and dopamine (Scott et al. 2008). In particular, CCK has been found to play a pivotal role in placebo analgesia and nocebo hyperalgesia. Its blockade by means of the non-specific CCK-1/CCK-2 receptor antagonist, proglumide, has been shown to increase the placebo analgesic response (Benedetti et al. 1995) and to block the nocebo hyperalgesic response (Benedetti et al. 1997, 2006). Although CCK blockade has furnished important information on the modulation of pain by placebos and nocebos, so far no study has investigated the effects of CCK activation. However, it should be noted that Gospic et al. (2008) found that pentagastrin and opioids have opposite effects on emotional processing. In fact, whereas pentagastrin increases the rating of unpleasantness for both neutral and unpleasant pictures and decreases the rating of pleasantness for neutral pictures, the opioid agonist remifentanil increases the pleasantness for neutral pictures, which indicates that CCK and opioids modulate how external stimuli, and not only noxious stimuli, are emotionally perceived.

The high inter-individual variability in placebo responsiveness may be attributable, among other factors, to a variation in the activity of these neurotransmitters. For example, the reduced activity or the complete blockade of μ-opioid receptors may lead to placebo unresponsiveness (Benedetti et al. 2007; Eippert et al. 2009a). Likewise, variations in dopamine D2–D3 receptor activity in the reward circuitry have been found to be related to placebo responsiveness, both in pain (Scott et al. 2007) and in other conditions, such as Parkinson's disease (de la Fuente-Fernandez et al. 2001). The present study shows that the disruption of placebo responses may also occur with the hyperactivity of CCK type-2 receptors. This finding underscores the central role of CCK in the modulation of pain by placebos and highlights the role of CCK type-2 receptors in complex cognitive/affective functions. Thus, CCK-2 receptor activity may be crucial in determining placebo responders and non-responders.

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Conflicts of interest The authors have no conflicts of interest.

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