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Research Paper

Morphine or its withdrawal affects plasma malondialdehyde, vitamin E levels and absence or presence of abstinence signs in rats

Arnaldo Pinelli[a], Giuliana Cighetti[b], Silvio Trivulzio[a] and Roberto Accinni[c]

[a]Department of Pharmacology, [b]Department of Medical Chemistry, University of Milan, Milan and [c]CNR Institute of Clinical Physiology, Ospedale Niguarda Ca' Granda, Milan, Italy

Abstract

Objectives Various experimental observations show that morphine treatment generates reactive oxygen species, and that its discontinuation leads to signs of withdrawal. We therefore investigated plasma malondialdehyde and vitamin E levels under both conditions to verify the occurrence of any alterations in oxidative metabolism, and whether these are associated with behavioural changes.

Methods We investigated the effects of morphine or morphine plus naloxone on plasma malondialdehyde, vitamin E levels and withdrawal signs such as jumping, wet dog shakes and faecal excretion in rats. Furthermore, isopropylnoradrenaline was injected in rabbits to verify its effects on plasma malondialdehyde levels.

Key findings Morphine treatment increased free malondialdehyde and decreased vitamin E levels. The elevation in malondialdehyde levels were exacerbated by the abrupt removal of morphine by naloxone, which also led to the appearance of withdrawal signs. The increased malondialdehyde values can be attributed to the interactions of reactive oxygen species with unsaturated fatty acids, and the lowered levels of vitamin E to its interactions with reactive oxygen species.

Conclusions A connection seems to exist between altered peroxide status and withdrawal signs in abstinent animals.

Keywords malondialdehyde (MDA); morphine; morphine withdrawal; reactive oxygen species (ROS); vitamin E

Introduction

Morphine and other related compounds interfere with oxido-reductive metabolism. Morphine stimulates the production of superoxide molecules in polymorphonuclear leucocytes[1] and glomerular mesangial cells,[2] and increases thiobarbituric acid reactive substances (TBARS) in human hepatocytes *in vitro*.[3]

Various authors have reported that morphine induces oxidative damage to biomolecules in mouse liver,[4] and that heroin causes oxidative stress conditions[5] or oxidative lesions[6] in mice. These studies also found that the administration of natural antioxidants had a protective effect. It has also been reported that morphine plus naloxone increases noradrenergic activity,[7] and that noradrenaline generates reactive oxygen species (ROS).[8]

As all of these findings indicate that ROS are involved in morphine treatment and withdrawal, it seems to be worth studying some aspects of oxidative stress using suitable markers. Oxidative stress leads to lipid peroxidation, with the production of lipid hydroperoxides and the generation of a number of aldehydes, including malondialdehyde (MDA),[9] a widely accepted oxidative marker,[10] which can be evaluated using an effective method[11] in the presence of morphine or morphine withdrawal.

Various authors have shown that morphine lowers antioxidative potential by decreasing the levels of reduced glutathione (GSH) in isolated hepatocytes or the caudatus nucleus in rats,[12] and in the cerebrospinal fluid of patients receiving intracerebroventricular morphine injections.[13] Others have found that opiate treatment or withdrawal depresses antioxidant status[6] by reducing total antioxidant capacity and antioxidative enzyme activity.[5] It therefore seems to be worth evaluating the effect of morphine or morphine plus naloxone on

Correspondence: Professor Arnaldo Pinelli, Department of Pharmacology, Via Vanvitelli 32, 20129 Milano, Italy.
E-mail: arnaldo.pinelli@unimi.it

antioxidant potential by measuring vitamin E levels.[14] As signs of abstinence can also be seen during the withdrawal of morphine,[7] it is also worth investigating whether they may be related to alterations in peroxide or antioxidant levels.

It has been reported that administration of morphine plus naloxone is associated with a considerable increase in noradrenergic activity and noradrenaline turnover.[7] Noradrenaline is an α- and β-agonist[15] that produces peroxide.[8]

To verify whether noradrenergic hyperactivation induces the biogenesis of MDA, we investigated whether injecting the pure β-agonist, isopropylnoradrenaline, was capable of causing MDA formation in rabbits.

In this study we describe the effects of morphine or morphine plus naloxone on plasma MDA, vitamin E levels and withdrawal signs, such as jumping, wet dog shakes and faecal excretion, in rats. Furthermore, isopropylnoradrenaline was injected in rabbits to verify its effect on plasma MDA levels.

Materials and Methods

Animal procedures

The experimental protocol and procedures were performed in accordance with Italian Legislative Decree No. 116 27/01/1992 and with the approval of the local University Committee on Laboratory Animals.

Rats

The rat studies involved male Sprague-Dawley rats, 270 ± 20 g (Harlan, Milan, Italy), which were housed in plastic cages and acclimatised to the laboratory conditions (temperature 22 ± 2°C; relative humidity 50 ± 5%; 12-h light–dark cycle) for seven days. Before and during the experiments, the rats were allowed free access to food and water.

Treatment

The morphine was supplied by S.A.L.A.R.S. S.p.A. (Como, Italy) and naloxone was purchased from Sigma (Milan, Italy). Both drugs were dissolved in saline solution.

The morphine was administered and withdrawn as previously described.[16,17] Morphine was administered intraperitoneally (i.p.) to 24 rats for four days in the form of three daily injections given at 150-min intervals at the following doses: 9, 16 and 25 mg/kg (day 1); 25, 25 and 50 mg/kg (day 2); 50, 50 and 50 mg/kg (day 3); and 50, 50 and 100 mg/kg (day 4). The morphine group was larger than the other groups as previous experience had shown that morphine treatment causes about 30% mortality. The rats died mostly during the second and third day of morphine treatment. Of the survivors, 12 were randomly selected and six received saline (M) and six received naloxone (M + N). Furthermore from a group of 24 rats not previously treated, two groups of six rats each were randomly chosen and each received saline alone (controls) or naloxone alone (N). Naloxone was given at a dose of 30 mg/kg intraperitoneally 180 min after the last morphine injection, only on the last day.

Behavioural signs and faecal excretion

The rats were placed in plastic cylinders (50 × 18 cm) over previously weighed filter paper dishes, and behavioural signs, such as jumping and wet dog shakes (which only appeared in the M + N group), were evaluated by counting the number of events occurring during the 30 min following naloxone injection. Faecal excretion was evaluated by weighing the stool on the paper dishes.

Blood sampling

Forty-five minutes after the naloxone injection (in those receiving it), the rat were sacrificed, and their blood was immediately collected using citrate 3.8 mg/ml. The plasma was prepared and stored at −20°C until assay.

Rabbits

Male New Zealand white rabbits, 3000 ± 100 g (Harlan, Milan, Italy), were divided into two groups of six rabbits each: one group received saline alone (controls) and the other was treated with isopropylnoradrenaline 3 mg/kg intraperitoneally. The rabbits were anaesthetised with urethane 700 mg/kg intraperitoneally, and clonazepam 0.25 mg/kg was given intraperitoneally 30 min later. The rabbits were sacrificed 60 min after isopropylnoradrenaline administration and their blood was immediately collected using citrate 3.8 mg/ml. The plasma was prepared and stored at −20°C until assay.

Biochemical parameters

Plasma levels of free MDA were assayed using the gas chromatography–mass spectrometry method described by Cighetti.[11] The free MDA was directly extracted from plasma without any hydrolytic procedure. The free MDA is reported in the text as MDA; plasma vitamin E was evaluated using an electrochemical detector after HPLC separation.[18]

Statistics

Plasma free MDA and vitamin E levels, and faecal excretion in the various groups were compared using analysis of variance, with Tukey's test being used to determine the statistical significance of the between-group differences. Statistical significance was assumed at: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.[19]

In addiction MDA levels were evaluated on the basis of the original 2 × 2 factorial design (morphine × naloxone) by means of factorial analysis using SAS software, version 9.1 (GLM procedure; SAS Institute, NC, US); the components were considered statistically significant at the level of 5%. The correlation between vitamin E and MDA was calculated using Pearson's correlation coefficient (r); statistical significance was set at $P = 0.005$.[19]

The number of jumping and wet dog shake events was recorded as mean values ± SEM.

Results

Rats

Plasma free MDA levels

The mean plasma free MDA level in the M group was 6.61 ± 0.9 μmol/l, significantly higher ($P < 0.01$) than that observed in the control (4.27 ± 0.24 μmol/l) group but MDA levels increased in the M + N group (9.88 ± 0.60 μmol/l) significantly ($P < 0.01$ vs the M group; $P < 0.001$ vs the control and N group) (Table 1).

Table 1 Levels of free plasma malondialdehyde in rats treated with morphine, naloxone, or both

Group	Treatment	Plasma malondialdehyde (μmol/l)
1	Control (C)	4.27 ± 0.24
2	Naloxone (N)	4.84 ± 0.35
3	Morphine (M)	6.61 ± 0.90
4	Morphine and naloxone (M + N)	9.88 ± 0.60

Values are presented as the mean ± SEM for six rats in each group. $P < 0.01$, group 1 vs group 3 and group 3 vs group 4; $P < 0.001$, group 1 vs group 4 and group 2 vs group 4.

Table 2 Analysis of variance in a factorial design 2 × 2 ± morphine ± naloxone: single effects of the factors morphine, naloxone, morphine × naloxone related to the levels of free plasma malondialdehyde

Source of variability	DF	Mean square	F	*P* values
Morphine	1	117.359	37.02	0.0001
Naloxone	1	34.011	10.73	0.0027
Morphine × Naloxone	1	15.392	4.86	0.0354

DF, degree of freedom; F, F ratio, Fischer test; *P* values, for the F ratio. Morphine × naloxone interaction is significant ($P < 0.05$).

Factorial analysis of the effects of morphine, naloxone, and morphine + naloxone on the levels of free MDA showed that the morphine × naloxone interaction was significant ($P < 0.05$) (Table 2).

Plasma vitamin E levels

Plasma vitamin E levels in the M group (8.05 ± 0.22 μmol/l) were significantly lower ($P < 0.01$) than in the controls (9.03 ± 0.23 μmol/l). The values detected in the M + N group (7.91 ± 0.35 μmol/l) were significantly reduced ($P < 0.01$ or $P < 0.05$) in comparison with controls or N group (9.03 ± 0.23 or 8.82 ± 0.29 μmol/l), respectively (Table 3). The MDA vs vitamin E scatterplot revealed a negative correlation between the two markers (Figure 1; $r = -0.466$, $P = 0.005$).

Behavioural signs and faecal excretion

Jumping and wet dog shakes were only observed in the M + N group; the mean number of events was, respectively, 10.88 ± 2.4 and 2.78 ± 0.43.

Faecal excretion was similar in the control, M and N groups but much greater in the M + N group than in controls: 4.89 ± 0.65 vs 0.51 ± 0.05 g.

Table 3 Levels of plasma vitamin E in rats treated with morphine, naloxone, or both

Group	Treatment	Plasma vitamin E (μmol/l)
1	Control (C)	9.03 ± 0.23
2	Naloxone (N)	8.82 ± 0.29
3	Morphine (M)	8.05 ± 0.22
4	Morphine plus naloxone (M + N)	7.91 ± 0.35

Values are presented as the mean ± SEM, for six rats in each group. $P < 0.05$, group 2 vs group 4; $P < 0.01$, group 1 vs group 3 and group 1 vs group 4.

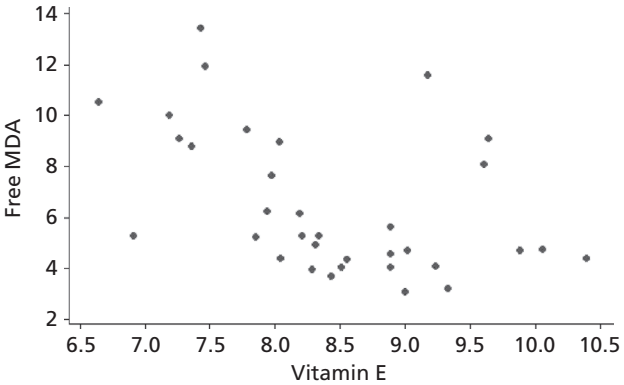

Figure 1 Scatterplot of malondialdehyde (MDA) vs vitamin E in plasma of rats treated with morphine naloxone or both shows a negative and significant correlation ($r = -0.466$; $P = 0.005$) between the two markers.

Rabbits

Plasma free MDA levels

Isopropylnoradrenaline treatment significantly increased free MDA levels in comparison with controls (4.15 ± 0.38 vs 1.93 ± 0.22 μmol/l; $P < 0.001$) (Table 4).

Discussion

Morphine treatment and withdrawal interfere with the plasma levels of oxidative and antioxidative markers, and the appearance of behavioural symptoms.

Administration with morphine alone increases free MDA levels in comparison with saline or naloxone treatment. Other authors have observed increased MDA or thiobarbituric acid reacting substances (TABRS) levels following opiate administration.[4–6] The increase in MDA is due to the capacity of morphine to generate toxic oxygen species.[1–3]

Free MDA levels were also different between the M and M + N groups, being markedly increased in the latter. Factorial analysis showed a significantly positive interaction ($P < 0.05$) between morphine and naloxone in relation to MDA levels, as shown in Table 2.

The displacement of morphine by naloxone induces noradrenaline activation:[7] noradrenaline favours the formation of hydroxyl radicals,[8] which initiate the oxidation of lipids by attacking the double bonds of unsaturated fatty acids and generate MDA.[9] We demonstrated here the involvement of catecholamines in MDA production by

Table 4 Levels of free plasma malondialdehyde in isopropylnoradrenaline-treated rabbits

Group	Treatment	Plasma malondialdehyde (μmol/l)
1	Control	1.93 ± 0.22
2	Isopropylnoradrenaline	4.15 ± 0.38***

Values are presented as the mean ± SEM for six animals in each group. ***$P < 0.001$ vs control.

injecting rabbits with isopropylnoradrenaline, which increases MDA levels.

Furthermore the ROS, generated in the M or M + N groups, may react with vitamin E, which scavenges hydroxyl radicals.[20] Vitamin E levels were decreased in our M and M + N groups and, as shown in Figure 1, negatively correlated with the levels of free MDA (Pearson's correlation coefficient $r = -0.466$; $P = 0.005$), thus further indicating the altered oxidative and antioxidative status of the rats in the M and M + N groups. The reduced antioxidant power in the rats treated with morphine or morphine + naloxone is in line with the findings of other authors, showing a decrease in reduced glutathione levels after morphine exposure,[12] or in antioxidant enzymes in mice treated with heroin.[4,6]

The M + N group also showed behavioural alterations, including jumping, wet dog shakes and a high level of faecal excretion. Other authors have described morphine withdrawal signs in the presence of altered oxidative metabolism, including animals showing jumping behaviour in association with a high dopamine turnover with peroxide biogenesis in the striatum,[21,22] or animals showing wet dog shakes in association with the activation of serotoninergic metabolism and peroxide production.[23–25] The increased faecal excretion in the M + N group may be due to peroxide facilitating cyclo-oxygenase and lipoxygenase activity,[26] and metabolites of arachidonic acid inducing intestinal secretion[27,28] or causing ileum contractions.[29,30]

There seems to be a relationship between high free MDA levels and the presence of withdrawal signs, detected in the morphine plus naloxone group. In morphine withdrawal conditions, some authors reported the presence of abstinence signs and noradrenaline hyperactivation.[7,31] Other authors described the peroxide generation by catecholamines: hydroxyl radicals by noradrenaline[8] and MDA by isopropylnoradrenaline demonstrated here. Noradrenergic hyperactivation in morphine-deprived rats is therefore the link capable of inducing both high peroxide levels and the appearance of withdrawal signs.

As lowered plasma antioxidant levels[32] and high plasma peroxide values[33] have been reported in heroin addicts showing withdrawal signs[34] and increased noradrenaline metabolites,[7] it may be rational to treat heroin addicts with a combination of antioxidants and anti-adrenergic agents.

Additional experiments with morphine + naloxone + anti-adrenergic drugs (alpha- and/or beta-blockers) are programmed, to prove our suggestion and to clarify the mechanism responsible for the observed effects in this animal model.

Conclusions

Treatment with morphine or morphine plus naloxone affects plasma peroxide levels and the absence or presence of withdrawal signs.

The link between peroxide generation and abstinence signs in the morphine plus naloxone group of animals seems to be attributable to noradrenergic hyperactivation.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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