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# Arachidonic acid and its metabolites are involved in the expression of morphine dependence in guinea-pig isolated ileum

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**Abstract**

The effects of phospholipase $A_2$, cyclooxygenase-1, cyclooxygenase-2 and 5-lipoxygenase inhibitors were investigated on the naloxone-precipitated withdrawal contracture of the acute morphine-dependent guinea-pig ileum in vitro. Mepacrine (a phospholipase $A_2$ inhibitor), tolmetin (selective cyclooxygenase-1 inhibitor) and meloxicam (selective cyclooxygenase-2 inhibitor) treatment before or after morphine was able to both prevent and reverse the naloxone-induced contracture after exposure to morphine in a concentration-dependent fashion. Also, nordihydroguaiaretic acid (5-lipooxygenase inhibitor) was able to block the naloxone-induced contracture following exposure to morphine when injected before or after the opioid agonist. The results of the present study suggest that arachidonic acid and its metabolites (prostaglandins and leukotrienes) are involved in the development of opioid withdrawal. © 1997 Elsevier Science B.V.

*Keywords:* Arachidonic acid; Opioid; Withdrawal; Ileum

## 1. Introduction

Dependence can be induced and measured in vitro by using guinea-pig ileum (Lujan and Rodriguez, 1981; Collier, 1980; Collier et al., 1981; Chal, 1983, 1986). Tissues from untreated animals, after a brief exposure to opioids, show a strong naloxone-induced contracture (Eisenberg, 1982; Valeri et al., 1990a,b; Morrone et al., 1990, 1993; Valeri et al., 1992) indicating that cellular mechanisms of dependence may occur very rapidly following occupation of receptors and that these mechanisms are operative within the myenteric plexus (North and Karras, 1978).

Recently, we have demonstrated that dexamethasone reduces opioid withdrawal through a protein synthesis-dependent mechanism via glucocorticoid receptors (Capasso et al., 1996).

In this respect, since prostaglandins have been reported to be involved in the expression of opiate withdrawal (Johnson et al., 1988) and glucocorticoids control prostaglandin and leukotriene biosynthesis by inhibiting the release of their common precursor, arachidonic acid through phospholipase $A_2$ inhibitory proteins (Gryglewski, 1976; Di Rosa and Persico, 1979; Blackwell et al., 1980; Dennis, 1987; Shimizu and Wolfe, 1990; Glaser et al., 1993; Barnes and Adcock, 1993), we suggested that dexamethasone could reduce opiate withdrawal by blocking the release of the prostaglandin precursor, arachidonic acid (Capasso et al., 1996). In fact, arachidonic acid is released by the enzyme phospholipase $A_2$ (Vane, 1971; Hirata et al., 1980; Carnuccio et al., 1981; Flower and Blackwell, 1979; Rothhut et al., 1983) and it is subsequently converted to prostaglandins and leukotrienes by the enzymes cyclooxygenase and lipoxygenase, respectively (Vane, 1971; Murphy et al., 1979; Dennis, 1987; Shimizu and Wolfe, 1990; Glaser et al., 1993).

Therefore, to verify whether the dexamethasone-induced reduction of opiate withdrawal is related to inhibition of the arachidonic acid cascade (Capasso et al., 1996), in the present work we have considered the effects of phospholipase $A_2$, cyclooxygenase and lipoxygenase inhibitors on morphine withdrawal.

Furthermore, although in a previous paper Johnson et al. (1988) reported that indomethacin, a well-known cyclooxygenase inhibitor, was able to reduce morphine withdrawal in vitro, we must remember that two isoform of cyclooxygenase exist: a constitutive (cyclooxygenase-1) form and an inducible (cyclooxygenase-2) form (Vane and Botting, 1995).

Cyclooxygenase-1 is constitutively expressed in most tissues and synthesizes prostaglandins which regulate nor-

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mal cell activity, whereas cyclooxygenase-2 is induced in a number of cells by pro-inflammatory stimuli (Vane, 1994; Vane and Botting, 1995). Selective cyclooxygenase-1 and cyclooxygenase-2 inhibitors have been reported (Vane and Botting, 1995). Indomethacin is a non-selective cyclooxygenase inhibitor since it blocks both cyclooxygenase-1 and cyclooxygenase-2 (Vane and Botting, 1995). Therefore, in the present work we used tolmetin as a selective cyclooxygenase-1 inhibitor and meloxicam as a selective cyclooxygenase-2 inhibitor (Vane and Botting, 1995) to verify whether the prostaglandins involved in opiate withdrawal are produced by cyclooxygenase-1 and/or cyclooxygenase-2. Finally, mepacrine was used as a phospholipase $A_2$ inhibitor (Vadas, 1982; Dennis, 1987) and nordihydroguaiaretic acid as a lipoxygenase inhibitor (Dennis, 1987; Shimizu and Wolfe, 1990; Glaser et al., 1993). All drugs were added before or after opiate agonists to verify whether they could act by preventing or reversing opiate dependence.

## 2. Materials and methods

### 2.1. Animals

Adult male guinea pigs (200–250 g) purchased from Charles River Italy were used in the experiments. Animal care and use followed the directions of the Council of the European Community (1986). The animals were housed in colony cages (4 guinea pigs each) with free access to food and water. They were maintained in a climate- and light-controlled room ($22 \pm 1$°C, 12/12 h dark/light cycle) at least 7 days before testing.

### 2.2. Preparation of guinea-pig isolated ileum

The animals were killed by $CO_2$ and bled. The terminal portion of the ileum, discarding the 10 cm nearest the caecum, was kept in a Petri dish with Tyrode solution (g/l: NaCl 8.00, KCl 0.20, $CaCl_2$ 0.20, $MgCl_2 \cdot 6H_2O$ 0.10, $NaH_2PO_4 \cdot H_2O$ 0.05, $NaHCO_3$ 1.00, glucose 1.00) for 30 min and then washed free of faecal matter. Segments, 2–3 cm long, from the same animal were placed between platinum electrodes and connected to a 85/2/50 model M.A.R.B. Stimulator (Ditta M.A.R.B., Chiesina Uzzanese, Pistoia, Italy). A force-displacement transducer and unirecord model polygraph were used for measurement of isotonic contractions (Ugo Basile, Milan, Italy). A resting tension of 0.5 g was applied. The bath solution was maintained at 37°C and continuously bubbled with a mixture of 95% $O_2$ and 5% $CO_2$.

### 2.3. Acute morphine dependence in guinea-pig isolated ileum

The experimental procedure was the same as described previously (Capasso et al., 1996). The ilea were allowed to equilibrate for 40–60 min without washing and the response to acetylcholine ($10^{-6}$ M) was determined three times so that responses could be expressed as percentage of the acetylcholine maximum response. Reproducible acute morphine dependence was obtained by performing the following experimental procedure. A typical tracing of contracture responses of the ileum to repeated challenges with morphine and naloxone is shown in Fig. 1. After three similar acetylcholine responses, the preparation was electrically stimulated for 10–20 min (0.5 ms pulse delivered transmurally, at a frequency of 0.1 Hz at supramaximal voltage of 25 V). Before the addition of morphine to the bath, the electrical stimulation was switched off. Under these conditions, the first contact with morphine followed by a 4-min exposure to naloxone induced a strong contracture (about 60% of the acetylcholine maximum) (Fig. 1A). After washout, another acetylcholine response was elicited (to verify whether ileum responsiveness was modified after withdrawal contracture) and, after a 30-min resting period under stimulation, a further 4-min exposure of the ileum (without electrical stimulation) to morphine and naloxone elicited a reproducible response (Fig. 1B). Following washout, the acetylcholine response (Fig. 1B) and another

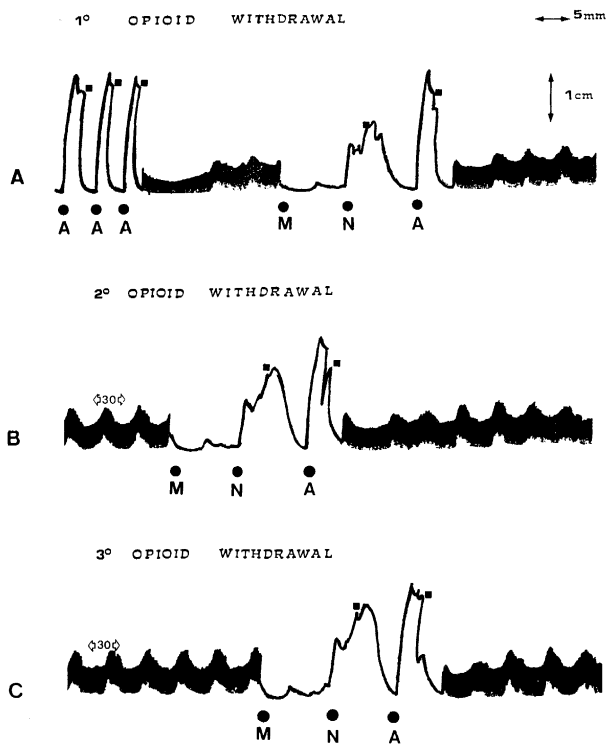


Fig. 1. Typical tracing of morphine withdrawal in guinea-pig ileum. (A) Three similar acetylcholine responses (A), electrical stimulation, injection of morphine (M) followed after 4 min by naloxone (N), which induces contraction (1st opioid withdrawal). After washout (■), another A response was elicited. (B) After a 30-min resting period under electrical stimulation, a further 4-min exposure of the ileum to M and N elicited a reproducible response (2nd opioid withdrawal). (C) After another 30-min resting period under electrical stimulation, the ileum responded again to the M and N with the same intensity (3rd opioid withdrawal).

30-min resting period under stimulation, the ileum responded again to morphine and naloxone with the same intensity (Fig. 1C). In our experiments, to avoid possible tolerance to repeated opiate injections, each preparation was submitted to only three challenges with the opioid agonist and naloxone.

Naloxone per se did not produce effects on 'naive' preparations or those washed after opiate contact.

### 2.4. Experimental procedure

The administration of mepacrine, tolmetin, meloxicam and nordihydroguaiaretic acid was performed according to the following schedule: (a) 3 acetylcholine responses; (b) electrical stimulation (10–20 min); (c) morphine administration in the absence of electrical stimulation (4 min) and addition of naloxone with subsequent contraction (1st opioid withdrawal); (d) washout and acetylcholine response; (e) electrical stimulation (30 min); (f) mepacrine, tolmetin and nordihydroguaiaretic acid ($1 \times 10^{-6}$–$5 \times 10^{-6}$–$1 \times 10^{-5}$ M) without electrical stimulation, added 10 min before or after morphine followed by naloxone (2nd opioid withdrawal); (g) washout and acetylcholine response; (h) electrical stimulation (30 min); (i) final control morphine withdrawal (3rd opioid withdrawal).

Meloxicam, injected before morphine, was used at concentrations of $1 \times 10^{-6}$–$5 \times 10^{-6}$–$1 \times 10^{-5}$ M whereas, when injected after morphine, it was used at concentrations of $1 \times 10^{-7}$–$5 \times 10^{-7}$–$1 \times 10^{-6}$ M.

Under our experimental conditions, to induce a strong contracture, morphine and naloxone were administered at the following concentrations: morphine ($10^{-5}$ M) + naloxone ($10^{-5}$ M).

Each experiment was performed with at least 6–9 isolated preparations from different animals.

### 2.5. Drugs

All drugs used in the experimental sessions were purchased from Sigma Chemical Co. (St. Louis, MO, USA) with the exception of morphine HCl, which was purchased from Carlo Erba (Milan, Italy), meloxicam, which was a generous gift from Dr. G. Cirino (Department of Experimental Pharmacology, University of Naples Federico II).

Drugs were dissolved in distilled water with the exception of meloxicam and nordihydroguaiaretic acid, which were dissolved in dimethyl sulfoxide (DMSO, Merck). DMSO per se did not affect opioid withdrawal.

### 2.6. Parameter evaluation

Four parameters were evaluated:

(1) Naloxone contracture: the size of the contracture produced by the naloxone challenge is expressed as a fraction of the maximum contraction obtained with the subsequent addition of acetylcholine to the same piece of tissue according to a modification of the method of Collier et al. (1981):

$$\frac{\text{Response to naloxone}}{\text{Maximum response to acetylcholine}} \times 100 = \text{Tension ratio}$$

(2) Acetylcholine responses before and after treatment: the decrease or increase in the acetylcholine responses in the post-drug period is expressed as a percentage of the acetylcholine response in the pre-drug period.

(3) Electrically stimulated contraction before and after treatment: the decrease or increase in the contraction following electrical stimulation in the post-drug period is expressed as a percentage of the electrical stimulation-induced contraction in the pre-drug period.

(4) Naloxone contraction before and after treatment: the decrease or increase in the naloxone contraction in the post-drug period was expressed as a percentage of the naloxone-induced contraction in the pre-drug period.

### 2.7. Statistical analysis

Results are expressed as means ± S.E.M. and were tested for statistical significance using Student's *t*-test for paired data when results before and after treatment on the same preparation were compared. $IC_{50}$ values and confidence limits (95%) were calculated with the method reported by Tallarida and Murray (1987).

## 3. Results

### 3.1. Concentration-related effect of mepacrine (phospholipase $A_2$ inhibitor) on morphine withdrawal

Mepacrine ($1 \times 10^{-6}$–$5 \times 10^{-6}$–$1 \times 10^{-5}$ M) administered 10 min before (or after) morphine induced a significant and concentration-dependent reduction of morphine withdrawal (Fig. 2). Mepacrine $IC_{50}$ was $9.2 \times 10^{-7}$ M

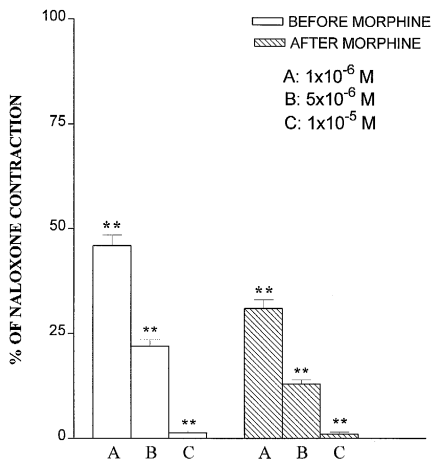


Fig. 2. Concentration-related inhibition by mepacrine of morphine withdrawal. Mepacrine was injected 10 min before or after the opioid receptor agonist. ** $P < 0.01$.

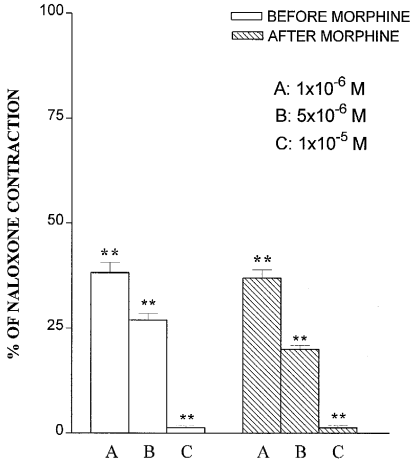


Fig. 3. Concentration-related inhibition by tolmetin of morphine withdrawal. Tolmetin was injected 10 min before or after the opioid receptor agonist. ** $P < 0.01$.

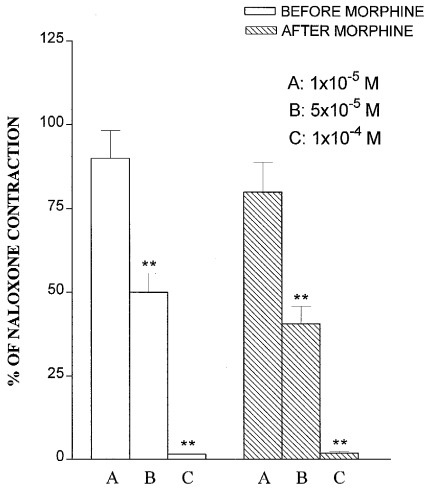


Fig. 5. Concentration-related inhibition by nordihydroguaiaretic acid of morphine withdrawal. Nordihydroguaiaretic acid was injected 10 min before or after the opioid receptor agonist. ** $P < 0.01$.

($5.6 \times 10^{-6}$–$1.5 \times 10^{-7}$) when it was injected before morphine and $3.5 \times 10^{-7}$ M ($5.0 \times 10^{-7}$–$2.5 \times 10^{-7}$) when injected after the opioid. After washout, both the acetylcholine-induced response and the electrical stimulation-induced response were not affected by mepacrine treatment whereas the final control morphine withdrawal response was still reduced (data not shown).

### *3.2. Concentration-related effects of tolmetin (selective cyclooxygenase-1 inhibitor), meloxicam (selective cyclooxygenase-2 inhibitor) and nordihydroguaiaretic acid (lipoxygenase inhibitor) on morphine withdrawal*

Tolmetin ($1 \times 10^{-6}$–$5 \times 10^{-6}$–$1 \times 10^{-5}$ M) added to the bath 10 min before (or after) morphine was strongly able to prevent and to reverse dose dependently morphine withdrawal (Fig. 3). Tolmetin $IC_{50}$ was $2.5 \times 10^{-7}$ M ($3.5 \times 10^{-6}$–$1.3 \times 10^{-7}$) when it was injected before morphine and $5.4 \times 10^{-7}$ M ($7.5 \times 10^{-7}$–$2.1 \times 10^{-7}$) when injected after the opioid. Meloxicam injected 10 min before morphine at concentrations of $1 \times 10^{-6}$–$5 \times 10^{-6}$–$1 \times 10^{-5}$ M was strongly able to reduce opiate withdrawal (Fig. 4), $IC_{50} = 6.9 \times 10^{-7}$ M ($4.3 \times 10^{-6}$–$1.1 \times 10^{-7}$).

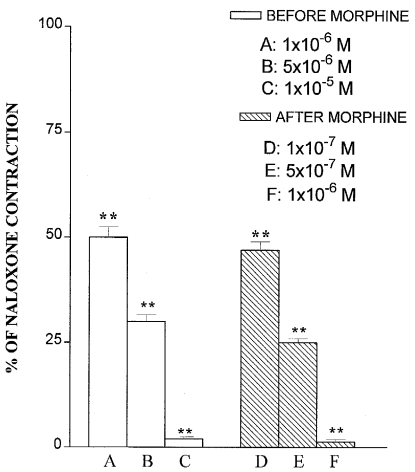


Fig. 4. Concentration-related inhibition by meloxicam of morphine withdrawal. Meloxicam was injected 10 min before or after the opioid receptor agonist. ** $P < 0.01$.

When the cyclooxygenase-2 inhibitor was added 10 min after morphine, the concentrations required to reduce opiate withdrawal were lower ($1 \times 10^{-7}$–$5 \times 10^{-7}$–$1 \times 10^{-6}$ M) (Fig. 4), $IC_{50} = 8.7 \times 10^{-8}$ M ($3.2 \times 10^{-7}$–$2.3 \times 10^{-8}$).

Also, nordihydroguaiaretic acid ($1 \times 10^{-6}$–$5 \times 10^{-6}$–$1 \times 10^{-5}$ M) added to the bath 10 min before (or after) morphine was strongly able to prevent and to reverse dose dependently morphine withdrawal (Fig. 5). Nordihydroguaiaretic acid $IC_{50}$ was $7.0 \times 10^{-6}$ M ($3.5 \times 10^{-6}$–$1.3 \times 10^{-7}$) when injected before morphine and $2.4 \times 10^{-6}$ M ($8.6 \times 10^{-5}$–$7.1 \times 10^{-6}$) when injected after the opioid.

After washout, both the acetylcholine-induced response and the electrical stimulation-induced response were not affected by tolmetin, meloxicam or nordihydroguaiaretic acid treatment whereas the final morphine withdrawal response was still reduced (data not shown).

## 4. Discussion

The present study indicates that mepacrine, a phospholipase $A_2$ inhibitor, strongly reduces morphine withdrawal, suggesting that during opiate withdrawal there may be a release of arachidonic acid after phospholipase $A_2$ activation.

This relationship between arachidonic acid and opioid withdrawal may be further supported by data showing that morphine is able to release arachidonic acid (Sergeeva et al., 1993).

The possible involvement of arachidonic acid in the development of opiate withdrawal is further supported by the results of experiments performed with cyclooxygenase and lipoxygenase inhibitors. In fact, tolmetin (cyclooxygenase-1 inhibitor), meloxicam (cyclooxygenase-2 inhibitor) and nordihydroguaiaretic acid (lipoxygenase inhibitor) were also able to reduce morphine withdrawal, indicating that arachidonic acid metabolites (prostaglandins and leukotrienes) are also involved in the expression of opiate withdrawal, as previously suggested (Capasso et al., 1996).

Although the role of prostaglandins in the expression of opiate withdrawal has already been determined in vitro by using indomethacin, a non-selective cyclooxygenase inhibitor (Johnson et al., 1988), this is the first paper which evaluates whether the prostaglandins involved in the development of opiate withdrawal are produced by the enzymes cyclooxygenase-1 and/or cyclooxygenase-2.

The results of our experiments indicate that both cyclooxygenase-1 and cyclooxygenase-2 are involved in the expression of opioid withdrawal since both tolmetin (cyclooxygenase-1 inhibitor) and meloxicam (cyclooxygenase-2 inhibitor) were able to reduce morphine withdrawal.

It is of interest to note that meloxicam (cyclooxygenase-2 inhibitor) compared to tolmetin (cyclooxygenase-1 inhibitor) appeared to be more active in inhibiting morphine withdrawal. In fact, when meloxicam was added after morphine, the concentrations required to reduce opiate withdrawal were lower than those of tolmetin. These findings may reflect differing affinities of the drugs for the enzymes or some other kinetic parameter.

These findings could also be explained on the basis of the different pharmacological profile of the enzymes cyclooxygenase-1 and cyclooxygenase-2 (Vane and Botting, 1995). In fact, cyclooxygenase-1 is a constitutive enzyme present in almost all cell types and regulates normal cell activity whereas cyclooxygenase-2 is effectively absent from healthy tissues but its level rises dramatically during tissue damage (Vane, 1994; Vane and Botting, 1995).

If we consider the opiate withdrawal to be a pathologic condition and morphine to be a noxious stimulus for the cell, the possibility that, during dependence, morphine induces also the activation of the enzyme cyclooxygenase-2 should be considered.

Although our data provide strong evidence that the prostaglandins involved in the development of opioid withdrawal are produced by both cyclooxygenase-1 and cyclooxygenase-2, the latter enzyme could represent the expression of pathologic conditions induced by morphine during the withdrawal phenomenon, and the strong activity of meloxicam supports this possibility.

Dexamethasone has been reported to block the induction of cyclooxygenase-2 (Vane and Botting, 1995). Therefore, the present data suggest that dexamethasone may inhibit prostaglandin production during opiate withdrawal (Capasso et al., 1996) also through the blockade of cyclooxygenase-2 activity.

We report the first evidence that lipoxygenase products are also involved in the development of opiate withdrawal since nordihydroguaiaretic acid strongly blocked the withdrawal phenomenon. Nordihydroguaiaretic acid is a drug routinely used as an inhibitor of lipoxygenase, although it has been shown to inhibit enzymes such as protein kinase C, cyclooxygenase, guanylate cyclase and cytochrome $P_{450}$ in various cell and tissue systems (Clark and Linden, 1986; Rondeau et al., 1990; Force et al., 1991). Furthermore, nordihydroguaiaretic acid inhibits $Ca^{2+}$ currents in some cell systems (Korn and Horn, 1990) and has some structural similarities with the sarcoplasmic reticulum $Ca^{2+}$-ATPase inhibitor, cyclopiazonic acid.

Therefore, the strong activity of nordihydroguaiaretic acid in reducing morphine withdrawal may be related to its inhibitory effects not only on lipoxygenase but also on cyclooxygenase or $Ca^{2+}$ currents in the cells. This last hypothesis should be considered since nifedipine, a calcium channel blocker, is able to block both the tolerance and physical dependence elicited by opioids (Valeri et al., 1990a).

Under our experimental conditions, each drug was able completely to inhibit morphine withdrawal, and although this effect could appear to be related to the higher concentrations used, analogous concentration-related inhibition has been shown in experiments performed in vitro (Vadas, 1982; Vane and Botting, 1995). In fact, the $IC_{50}$ value calculated for each drug in our study may be considered comparable to that reported previously (Vadas, 1982; Vane and Botting, 1995).

Taken together, the results of the present study confirm and extend those of our previous study performed with dexamethasone (Capasso et al., 1996), indicating that arachidonic acid and its metabolites (prostaglandins and leukotrienes) are involved in the development of opiate withdrawal. Furthermore, the ability of meloxicam to inhibit morphine withdrawal indicates that the enzyme cyclooxygenase-2 is induced by opiate withdrawal.

The physio-pathological significance of arachidonic acid metabolism in opiate dependence may be relevant considering the several diseases related to drug abuse. For example, one of the most important diseases associate with opiate dependence is the alteration of the immune system. It has been reported that morphine's immunosuppressive activity may be related to its ability to release arachidonic acid (Sergeeva et al., 1993), since an increase in phospholipase $A_2$ predisposes to an impaired defense against infections (Pruzanski et al., 1991).

Given the relationship between opiate abuse, arachidonic acid metabolism and immunodeficiency, our data appear to indicate that drugs effective in inhibiting the arachidonic acid cascade may control both opiate dependence and immunodeficiency related to opioids. Only clinical experiments can provide support for this hypothesis.

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