EFFECTS OF ACUTE AND CHRONIC MORPHINE TOLERANCE ON THE SERUM COPPER, CERULOPLASMIN AND HEPATIC COPPER CONTENTS

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Former papers from this laboratory reported that on treatment with sodium diethyl-dithiocarbamate (DDC), a dopamine-β-hydroxylase inhibitor and a chelating agent for copper, the analgesic action of morphine was potentiated in rats (1, 2). Although the possibility that effect of DDC on morphine analgesia might be related to catecholamine metabolism cannot be completely excluded, we clarified that the changes in copper metabolism of the blood and tissues in rats could be responsible for the potentiation of morphine analgesia by DDC and that fluctuation of the copper metabolism in rats by the treatment of morphine may be involved in the manifestation of analgesia and development of tolerance to the analgesia (2, 3).

Some authors have postulated that the development of acute and chronic tolerance to the analgesic action of morphine in rats appears to involve RNA and/or protein synthesis (4-6), because tolerance is prevented by drugs which inhibit RNA or protein synthesis.

The increase in the ceruloplasmin content in the serum, due to its synthesis in the liver after injection of copper, was also inhibited by actinomycin D and cycloheximide (7). Since the plasma copper content increases in animals which are tolerant to morphine analgesia (2), it seemed interesting to examine the effects of repeated intravenous injections or chronic treatment with morphine on the content of the serum copper protein, ceruloplasmin, during development of tolerance to the analgesic action of morphine. The present paper reports changes in the levels of serum copper, ceruloplasmin, hepatic copper and biliary excretion of copper in rat tolerated to the analgesic action of morphine caused by repeated intravenous or by chronic subcutaneous injections of morphine (acute or chronic morphine tolerant rat, respectively).

METHODS AND MATERIALS

Male Sprague-Dawley rats, weighing 140–180 g, were maintained on normal laboratory chow (type MF, Oriental Kobo Co.,) and tap water, and kept in individual wire cages in a room at 20±1°C. Chronic morphine tolerant rat was prepared as described previously (2). To prepare the acute morphine tolerant rat the method of Cox et al. (5) was slightly modified: morphine hydrochloride (morphine) (0.54 mg/kg/0.1 ml) was injected repeatedly
at 5 minutes intervals without anesthesia or detention into a polyethylene cannula inserted into the cervical vein. Analgesia was monitored at 30 minutes intervals by pinching both sides of the tail. The serum ceruloplasmin content, which is expressed as p-phenylenediamine oxidase activity, was measured as described by Houchin (8). The serum copper content was measured by the method of Landers and Zak (9). A polyethylene tube was inserted into the bile duct under ether anesthesia and bile fluid was collected for 4 hours after recovery from anesthesia. The copper content in the bile fluid was determined by atomic absorption spectrophotometry (Hitachi-Perkin-Elmer 303) after diluting the bile 2 fold with distilled, deionized water. To estimate the copper content in the liver, a modification of the method of Landers and Zak (9) was used after wet ashing as follows: Two tubes were used for each sample. An aliquot of 2.0 ml of the sample, 0.2 ml of saturated sodium pyrophosphate solution and 1.0 ml of 4 per cent hydroxylamine solution were put into one tube. Then, concentrated ammonium hydroxide was added dropwise until the pH of the mixture was 4–5, using methyl red as indicator. The same amount of sample, sodium pyrophosphate, and hydroxylamine and concentrated ammonium hydroxide were put into the second tube. The optical density of the latter mixture was measured at 480 nm against a reagent blank after addition of 1.0 ml of 0.28 % of bathocuproine in 40 % of sodium acetate solution. For subcellular fractionation, the method of Evans et al. (10) was used. The copper contents of the pellets and the final supernatant were measured by the above method after wet ashing. Judging from the DNA and RNA contents and succinic dehydrogenase activity of each fraction, none of the fractions seemed to be appreciably contaminated.

RESULTS

1. Effect of chronic morphine treatment on the levels of serum copper and ceruloplasmin and of hepatic copper

The copper content of the serum of animals with chronic morphine tolerance increased in parallel with a marked rise in the ceruloplasmin level while the copper content of the liver remained at the control level (Table 1).

| Treatment | Serum copper content (µg/dl) | Ceruloplasmin (O.D. units/ml) | Hepatic copper content (µg/g) |
|-----------|-----------------------------|-------------------------------|-----------------------------|
| Saline    | 121.0 ± 7.0<sup>a</sup>     | 1.52 ± 0.12                  | 5.25 ± 0.37                 |
| Morphine<sup>a</sup> | 243.3 ± 4.0<sup>c</sup>   | 2.77 ± 0.12<sup>c</sup>      | 5.33 ± 0.50                 |

<sup>a</sup>: Blood was collected 4 hours after the last injection of schedule dose of morphine (300 mg/kg, s.c.).
<sup>b</sup>: Values are means ± standard errors. Each group contained 14 animals.
<sup>c</sup>: Significantly different from the control (P < 0.01).

2. Development of tolerance to morphine analgesia by repeated intravenous injections of morphine, and effects of levallorphan and DDC on this tolerance

Repeated intravenous injections of morphine into rats (0.54 mg/kg/0.1 ml) at 5 minutes intervals resulted in a reduction in sensitivity to a tail pinch stimulus 30 minutes after the
start of the injections. About 5 hours after the beginning of the repeated injections of morphine, the sensitivity gradually returned to the normal level, with increase in appetite and in movement of the body. When a second series of intravenous injections of morphine was started 17 hours after termination of the first, the level of tolerance attained by the first series of injections was maintained (Fig. 1). When levallophan tartrate (levallorphan) (0.17 mg/kg/0.1 ml) was injected with morphine, the analgesic action of morphine was completely blocked. When DDC (8.3 mg/kg/0.1 ml) was injected with morphine, no tolerance to the analgesic action of morphine developed within 7 hours.

![Time of repeated injection of drugs](image)

**FIG. 1.** Development of tolerance to morphine on repeated intravenous injections of morphine and effects of levallorphan and DDC on development of tolerance.

Repeated intravenous injections of morphine (0.54 mg/kg/0.1 ml) were given at 5 minutes intervals. Levallorphan (0.17 mg/kg/0.1 ml) or DDC (8.3 mg/kg/0.1 ml) was administered simultaneously with morphine. A second series of injections of morphine was given 17 hours after termination of the first series. Control animals received saline. Groups receiving morphine with levallorphan or with DDC contained 8 animals each. Other groups contained 14 rats each. Analgesia was estimated by the tail pinch method.

**TABLE 2.** Effect of repeated intravenous injections of morphine on the levels of serum copper and ceruloplasmin, and effects of levallorphan and DDC on the response.

| Treatment                  | Copper content in serum (µg/dl) | Ceruloplasmin (O.D. units/ml) | Number of animals |
|----------------------------|---------------------------------|-----------------------------|-------------------|
| Saline                     | 128.9 ± 3.6                     | 1.60 ± 0.06                 | 14                |
| Morphine, 7 hr             | 168.7 ± 10.4*                   | 1.95 ± 0.10*                | 12                |
| Morphine + DDC, 7 hr       | 75.4 ± 11.6*                    | 0.70 ± 0.13*                | 7                 |
| Morphine + Levallophan, 7 hr | 140.2 ± 8.1                     | 1.80 ± 0.18                 | 5                 |

The dosages of drugs were as for Fig. 1. Values are means ± standard errors. *= Significantly different from the control (P<0.01).

3. **Effects of repeated intravenous injections of morphine on the levels of serum copper and ceruloplasmin, and the effects of levallorphan and DDC on the response***

Rats which were tolerant to morphine analgesia had higher serum copper and ceruloplasmin contents than control animals which had received saline injections for 7 hours.
(Table 2). When levallorphan was injected simultaneously with morphine, the serum copper
and ceruloplasmin contents were similar to those of control rats. When DDC was injected
with morphine, no tolerance to the analgesic action of morphine developed within 7 hours,
and the contents of serum copper and ceruloplasmin were about half the control values.

4. Correlation between the serum copper content and serum p-phenylenediamine oxidase
(ceruloplasmin) activity in vivo

A definite parallel was found on plotting the serum p-phenylenediamine oxidase activities and the corresponding copper contents of samples measured in this work (Fig. 2).

![Graph showing correlation between serum copper content and p-phenylenediamine oxidase activity.]

**TABLE 3. Effects of morphine, levallorphan and DDC on serum p-phenylenediamine oxidase activity in vitro.**

| Drugs     | Concentration (M) | Inhibition (%) | Activation (%) |
|-----------|-------------------|----------------|---------------|
| Morphine  | $1 \times 10^{-4}$ | 3              |               |
|           | $1 \times 10^{-2}$ | 1              |               |
| Levallorphan | $5 \times 10^{-4}$ |               | 1             |
| DDC       | $1 \times 10^{-4}$ | 24             |               |
|           | $1 \times 10^{-2}$ | 96             |               |

5. Effects of morphine, levallorphan and DDC on the serum p-phenylenediamine oxidase (ceruloplasmin) activity in vitro

Since the concentration of serum ceruloplasmin was measured as the p-phenylenediamine oxidase activity, we tested to see whether this could be partly due to activation of the oxidase by morphine. Morphine and levallorphan did not affect the oxidase activity, though DDC inhibited the activity markedly in vitro (Table 3).

6. Effect of repeated intravenous injections of morphine on the hepatic copper content

Three hours after the beginning of repeated intravenous injections of morphine, the hepatic copper content was increased and 4 hours later, the accumulated copper had de-
TABLE 4. Effect of repeated intravenous injections of morphine on the hepatic copper content and effects of levallorphan and DDC on the response.

| Treatment                        | Copper content of liver (µg/g) | Number of animals |
|----------------------------------|-------------------------------|------------------|
| Saline                           | 5.25±0.37                     | 14               |
| Morphine, 3 hr                   | 6.11±0.12*                    | 5                |
| Morphine, 7 hr                   | 5.44±0.22                     | 12               |
| Morphine + DDC, 7 hr             | 7.87±0.63*                    | 7                |
| Morphine + Levallorphan, 3 hr    | 5.15±0.49                     | 5                |

Dosages of drugs were as for Fig. 1. Values are means±standard errors.

*: Significantly different from the control, (P<0.01).

creased to the control value (Table 4). This accumulation was inhibited by injection of levallorphan with morphine. When DDC was administered with morphine, the copper content of the liver after 7 hours was much higher than that in other groups.

7. Subcellular distribution of the copper in the liver after the repeated intravenous injections of morphine

The elevation in the copper content of the liver, after the repeated intravenous injections of morphine for 3 hours, was mainly due to increase in copper in the microsomal and supernatant fractions. Then 4 hours after the injections, the copper contents of the microsomal and supernatant fractions returned to the control values. In animals which received repeated injections of both morphine and DDC, the copper level increased not only in the microsomal and the supernatant fractions, but also in the nuclear and large granular fractions (Table 5).

TABLE 5. Changes in subcellular distribution of copper in the liver by repeated intravenous administration of morphine and with DDC.

| Treatment                        | Copper concentration (µg/g fresh liver) |
|----------------------------------|----------------------------------------|
|                                 | Nuclei                   | Large granule       | Microsomes   | Supernatant |
| Saline                           | 0.82±0.03                 | 1.06±0.06           | 0.44±0.04    | 1.74±0.16   |
| Morphine, 3 hr                   | 1.00±0.15                 | 1.21±0.12           | 0.56±0.03*   | 2.85±0.58** |
| Morphine, 7 hr                   | 1.01±0.17                 | 0.96±0.04           | 0.42±0.04    | 1.71±0.11   |
| Morphine + DDC, 7 hr             | 1.22±0.11*                | 1.33±0.10*          | 0.54±0.03*   | 2.95±0.17** |

Subcellular fractionation was done by the method of Evans et al. (10).

Values are expressed as means±standard errors. Each group consisted of 6 animals.

Significance of difference from control, + : P<0.05 ; ++P<0.01.

8. Effects of repeated intravenous injections of morphine and with DDC, and chronic treatment with morphine on the excretion of copper in the bile

The changes in the biliary excretion of copper for 4 hours after the drug administration were studied. By the repeated intravenous injections of morphine, the amount of the copper excreted into the bile was greater than that by saline injection (P<0.05). This increment was observed 3–4 hours after the repeated injections of morphine, about 130% of control.
FIG. 3. Effects of the repeated intravenous injections of morphine and with DDC, and chronic treatment with morphine on excretion of copper in the bile.

From top to bottom the columns show the effects of repeated intravenous injections of morphine (0.54 mg/kg/0.1 ml), morphine with DDC (8.3 mg/kg/0.1 ml), and chronic morphine treatment.

By injection of morphine with DDC, copper excretion in the bile was much lower than by saline injection (P<0.01). On the contrary, in chronic morphine tolerant rat, copper excretion in the bile was lower than that of control animals (P<0.05) (Fig. 3).

DISCUSSION

In this work, the serum copper content of chronic morphine tolerant rat was found to increase in parallel with the increment in the level of the serum copper protein, ceruloplasmin. In acute morphine tolerant rat, the serum copper and ceruloplasmin levels increased but less than in chronic morphine tolerant rat.

The concentration of serum ceruloplasmin was measured as p-phenylenediamine oxidase activity. Thus it was possible that direct activation of the oxidase by morphine might contribute to the observed increase in the serum ceruloplasmin level. However, morphine did not affect the oxidase activity in vitro. Moreover, the serum oxidase activity increased in parallel with the increase in the serum copper content in vivo. Thus it seems that ceruloplasmin protein itself increased with increase in tolerance to the analgesic action of morphine.

Increase in the copper content of the liver on copper injection was reported to be followed by induction of serum ceruloplasmin (7) and Owen and Hazelrig (11) reported that ceruloplasmin was synthesized in microsomes.

So, the accumulation of copper in the microsomal fraction of the liver, which was evoked by repeated injections of morphine for 3 hours, might be responsible for the elevation of the serum ceruloplasmin content 7 hours after the start of the injection. However, on injection of morphine with DDC the microsomal fraction had the same copper content as on injection of morphine alone, though the serum copper and ceruloplasmin contents were very low. The exact explanation of these results is not clear at present.

Thus, in rats with acute morphine tolerance, the elevated copper content of the liver is partly reduced by increased incorporation of copper into ceruloplasmin for 7 hours after morphine injection. An alternative pathway of removal of copper seems to be excretion in the bile as Hazelrig et al. (12) demonstrated in normal rat liver.
So, blockage of biliary excretion in some way may result in increase in the hepatic copper content. This is supported by the observation of Dempsey et al. (13) that obstruction of the bile duct in rats caused an increase in the hepatic copper content. It is also supported by our finding that in rats receiving morphine with DDC, biliary copper excretion was much lower than that of control rats, while the copper content of the liver was much higher (Fig. 3., Table 4).

With regard to the form of the copper in the liver, it seems that metal incorporated into ceruloplasmin which is synthesized in microsomes of the liver (11) migrates into the bloodstream but not into the bile (14). On the other hand, copper bound with protein of low molecular weight, in the supernatant fraction of the liver is thought to give rise to biliary copper (15). When DDC was administered with morphine, the changes in copper metabolism, including biliary excretion of copper were opposite from those on injection of morphine alone. Thus these effects of DDC may be related to the find that tolerance to morphine analgesia did not develop within 7 hours by the simultaneous injection of DDC and morphine.

SUMMARY

In experiments on acute tolerance to analgesic action of morphine caused by repeated intravenous injections of the drug, tolerance developed within 7 hours. By repeated intravenous injections of morphine, the hepatic copper content showed an increase and then returned to the control value, with concomitant increase in the serum copper and ceruloplasmin contents and biliary excretion of copper. Examination of the subcellular distribution of copper in the livers of rats 3 hours after morphine treatment revealed that the copper content was mainly elevated in the microsomal and supernatant fractions. This elevation was antagonised by levallorphan. When DDC was administered with morphine, acute tolerance was not seen and the changes in copper metabolism, including biliary excretion, were opposite of those observed after the administration of morphine alone. In rats which had developed tolerance to the morphine analgesia by chronic administration of the drug, the serum copper and ceruloplasmin levels were markedly raised but the hepatic copper content remained at the control level. However, excretion of copper into the bile was low. These results suggest that copper and/or ceruloplasmin metabolism are involved in morphine analgesia and its tolerance.

REFERENCES

1) WATANABE, K., MATSUI, Y. AND IWATA, H.: Experientia 25, 950 (1969)
2) IWATA, H., WATANABE, K. AND MATSUI, Y.: European J. Pharmac. 11, 298 (1970)
3) IWATA, H., WATANABE, K., MIICHI, H. AND MATSUI, Y.: Pharm. Res. Comm. 2, 213 (1970)
4) COHN, M., KEATS, A.S., KRIVOY, W. AND UNGER, G.: Proc. Soc. exp. Biol. Med. 119, 381 (1965)
5) COX, B.M., GINSBURG, M. AND OSMAN, O.H.: Br. J. Pharmac. Chemother. 33, 245 (1968)
6) COX, B.M. AND OSMAN, O.H.: Br. J. Pharmac. Chemother. 38, 157 (1970)
7) EVANS, G.W., MAJORS, P.F. AND CORNATZER, W.E.: Biochim. biophys. Res. Commun. 41,
8) Houchin, O.B.: *Clin. Chem.* 4, 519 (1958)
9) Landers, J.W. and Zak, B.: *Am. J. clin. Path.* 29, 590 (1958)
10) Evans, G.W., Myron, D.R., Cornatzer, N.F. and Cornatzer, W.E.: *Am. J. Physiol.* 218, 298 (1970)
11) Owen, C.A. Jr. and Hazelrig, J.B.: *Am. J. Physiol.* 210, 1059 (1966)
12) Hazelrig, J.B., Owen, C.A. Jr. and Ackerman, E.: *Am. J. Physiol.* 211, 1075 (1966)
13) Dempsey, H., Cartwright, G.E. and Wintrobe, M.M.: *Proc. Soc. exp. Biol. Med.* 99, 67 (1958)
14) Evans, G.W. and Cornatzer, W.E.: *Proc. Soc. exp. Biol. Med.* 136, 719 (1971)
15) Worwood, M. and Taylor, D.M.: *Biochem. Med.* 3, 105 (1969)