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STRESS- AND MORPHINE-INDUCED ELEVATIONS OF PLASMA AND TISSUE CHOLESTEROL IN MICE: REVERSAL BY NALTREXONE

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Abstract—Our earlier studies indicated that stress-induced facilitation of gallstone formation could be prevented by the opiate antagonist naltrexone. In view of the possible link between gallstone formation and atherosclerosis, the present study examined the possibility that endogenous opioids might also mediate stress-induced hypercholesterolemia. A 28-day immobilization stress schedule was used to induce increases in plasma, aortic and liver cholesterol of mice maintained on a high cholesterol diet. These stress-induced increases in plasma, hepatic and aortic cholesterol were reversed by pretreatment with the opiate antagonist, naltrexone (1 mg/kg). Exposure of mice to morphine (0.1% in the drinking water for 28 days) resulted in elevations of plasma, liver, and aortic cholesterol levels, similar to those observed following immobilization. In contrast, chronic exposure to the peripherally restricted opiate agonist, loperamide (0.1% in the drinking water for 28 days), was ineffective. The antagonism by naltrexone and duplication by morphine but not loperamide suggest that stress-induced hypercholesterolemia may require the activation of central endogenous opioid systems.

A number of studies have demonstrated a relationship between stress and plasma or tissue cholesterol levels. In humans, preoperative stress is associated with an elevation of serum cholesterol [1], and stress is included among the risk factors for atherosclerosis in humans [2]. Elevation of both plasma and tissue cholesterol has been reported in animals exposed to footshock [3] and psychological stress [4]. The aorta appears to be particularly vulnerable to the effects of stress on cholesterol deposition [5]. Neural factors may be involved since electrical stimulation of the lateral hypothalamus in rats increases plasma cholesterol and induces changes in the arterial wall indicative of atherosclerosis [6].

Stress is also a well established activator of the endogenous opioid system [7]. Different stressors are known to release endorphin and ACTH from the pituitary [8] and enkephalin from the adrenals [9]. Endogenous opioids have been implicated in various stress-related phenomena, such as stress-induced eating [10, 11], stress-induced salt preference [12] and stress-induced increase in fat intake [13]. Additionally, opiate antagonists block stress-induced analgesia [14], stress-induced gastric ulcers [15], hypovolemic shock [16] and stress-induced gallstone development [17]. Interestingly, gallstone disease and atherosclerosis have been linked etiologically [18].

The aim of this study was to assess the possible role of endogenous opioids in stress-induced elevation of plasma and tissue cholesterol. Our previous experience indicated the ability of chronic, 28-day immobilization stress or morphine regimens to augment gallstone production in mice [17]. Accordingly, we examined the effects of chronic immobilization stress or morphine treatment on cholesterol homeostasis, and the ability of the opiate antagonist, naltrexone, to reverse the effects of stress and of chronic morphine treatment. The effects of the peripherally restricted opiate agonist, loperamide, were also studied.

METHODS

Subjects and general conditions. Female albino Swiss–Webster mice (Laboratory Supply Co. Inc., Indianapolis, IN) were housed individually in plastic cages (29 × 18 × 13 cm) with stainless steel tops. The lighting schedule was maintained on a 14 hr light/10 hr dark cycle (lights on at 6:00 a.m.; off at 8:00 p.m.), and room temperature was kept at 22°. The animals had free access to a powdered laboratory chow diet (Wayne) and tap water for a 7-day acclimation period prior to experimental manipulation. Following the acclimation period, the animals were fed the powdered diet to which 1.0% (w/w) cholesterol and 0.5% (w/w) cholic acid (Sigma Chemical Co., St. Louis, MO) had been added. The high cholesterol diet was utilized in order to maximize observed differences in plasma and tissue cholesterol levels. Cholic acid was added to reduce conversion of cholesterol to bile acid in the liver [19] as well as to facilitate cholesterol absorption via the formation of micelles [20].

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Chronic stress regimen. Mice maintained on the cholesterol–cholic acid diet were subjected to an unpredictable immobilization stress schedule. Each bout of stress was of 4-hr duration, and the average between-stress interval was 48 hr over a 28-day period (fourteen total stress periods). The mice were immobilized by placing them in plastic centrifuge tubes with ventilation holes. Four groups of mice (N = 7 to 11 per group) were utilized: control (cholesterol–cholic acid diet only), stress, stress plus naltrexone hydrochloride (1.0 mg/kg, s.c., administered 15 min prior to each stress session; Endo Laboratories Inc., Garden City, NY), and naltrexone only. Body weight and fluid intake were assessed regularly. Following the 28-day stress period, free-flowing blood samples were obtained by orbital sinus puncture and collected into chilled test tubes containing 1% EDTA. Centrifuged plasma samples were stored at −70° until the time for cholesterol assay. The mice were then killed by cervical dislocation, and the abdominal aorta and liver were removed and cleaned of connective tissue. Tissue samples were frozen at −70° for later analysis of cholesterol content.

Administration of opiates. The effect of chronic opiate treatment in mice on the high cholesterol diet was determined by the administration of morphine sulfate (Merck Chemical, Rahway, NJ) or loperamide hydrochloride (Ortho Pharmaceutical, Raritan, NJ) in the drinking water. The initial dose of either morphine or loperamide was 0.1 mg/ml; this was increased to 0.3 mg/ml of day 4 and 1.0 mg/ml (0.1%) on day 11 for the remainder of the 28-day treatment period. This method induces dependence in mice and produces diarrhea and jumping behavior following injection of an opiate antagonist [21]. Control mice received tap water. Fluid consumption was measured every day for each animal by using 5-ml pipettes adapted to drinking spouts. Body weight was determined every 3 days. Four groups of mice (N = 7 to 13 per group) were utilized: control (cholesterol–cholic acid diet only), morphine, morphine plus naltrexone (1.0 mg/kg, s.c., administered three times per week), and loperamide. Plasma and tissue samples were obtained following the treatment period as previously described and stored at −70° for later cholesterol analysis.

Cholesterol and lipoprotein analyses. Plasma cholesterol was determined according to the method of Rudel and Morris [22]. Ten-microliter samples of plasma were saponified with potassium hydroxide and ethanol. The cholesterol was then extracted into hexane, the hexone evaporated, and the cholesterol reacted with *o*-phthaldialdehyde; absorbance was read at 550 nm.

Liver and aortic cholesterol levels were determined using the same method after the tissue samples were homogenized for lipid extraction. One gram of liver was homogenized in 15 ml chloroform : methanol (2 : 1) and filtered with suction. This process was repeated twice with the residue, and the combined filtrate was diluted to 50 ml. One milliliter of this extract was dried under nitrogen and then saponified, and the cholesterol content was determined as described above. The whole aorta samples were weighed (average weight approximately 3 mg) and homogenized with 2 ml of the 2:1 chloroform : methanol mixture. This process was repeated twice after filtration, and the combined filtrate was evaporated under nitrogen and then saponified, as previously described.

Statistical analyses. Treatment groups were compared for significant differences using one-way analysis of variance followed by application of the Newman–Keuls range test when significant differences were indicated. Differences between groups were considered significant at a P value of less than 0.05.

RESULTS

As depicted in Fig. 1, chronic immobilization stress was associated with a significant increase in plasma cholesterol levels (43%). This elevation of total plasma cholesterol was prevented by naltrexone pretreatment, although mean plasma cholesterol levels in immobilized/naltrexone-treated mice were 14% higher than control. This difference was not statistically significant.

Immobilization stress also resulted in increases in the cholesterol concentration of both the liver and aorta (54 and 65% increase respectively). The stress-induced elevation of liver and aortic cholesterol was reduced by naltrexone pretreatment. However, blockade by the antagonist was only partial, as tissue cholesterol levels were still significantly greater (22 and 19% respectively) than those of control animals (Fig. 1). While opiate receptor blockade with naltrexone reduced stress-related increases in plasma and tissue cholesterol, naltrexone treatment alone had no effect on basal cholesterol levels in the plasma, liver or aorta (Fig. 1).

The effects of stress and naltrexone on plasma and tissue cholesterol levels were not associated with differences in body weight, as the rate of body weight

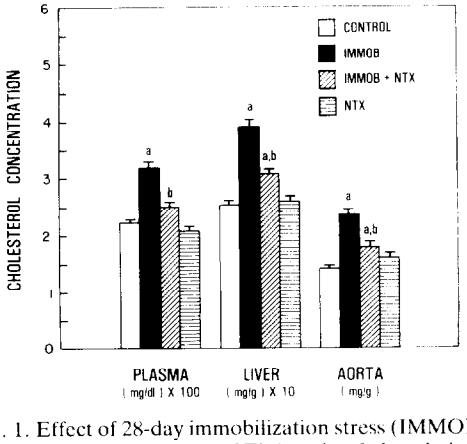

Fig. 1. Effect of 28-day immobilization stress (IMMOB) on cholesterol levels (mean ± SE) in mice fed a cholesterol (1.0%)–cholic acid (0.5%) diet. Control mice received the diet only; the IMMOB + NTX group received naltrexone (1.0 mg/kg, s.c.) 15 min prior to each stress period; the NTX group was maintained on the diet and received the naltrexone injections without immobilization. Key: (a) $P < 0.05$ vs control, and (b) $P < 0.05$ vs stress.

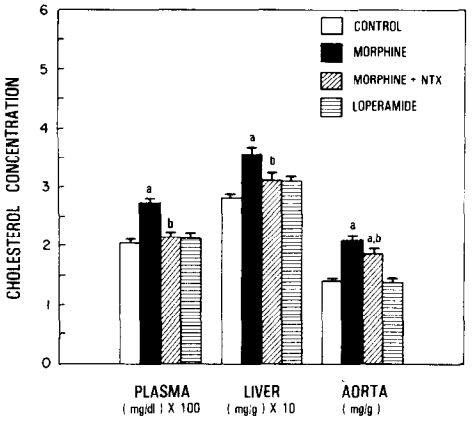

Fig. 2. Effect of 28-day morphine or loperamide (0.1% in the drinking water) on cholesterol levels (mean ± SE) in mice fed a cholesterol (1%)–cholic acid (0.5%) diet. Control mice received the diet only and tap water; the morphine + NTX group also received naltrexone (1.0 mg/kg, s.c., three times per week). Key: (a) $P < 0.05$ vs control, and (b) $P < 0.05$ vs morphine.

gain did not differ among the four treatment groups (data not shown).

Chronic morphine treatment (0.1% in the drinking water) led to a 34% elevation of total plasma cholesterol (Fig. 2), and this action of morphine was prevented by periodic administration of naltrexone. The chronic morphine regimen was also associated with elevations of liver (26%) and aortic cholesterol (48%). Periodic administration of naltrexone prevented the elevation of liver cholesterol with morphine. The morphine-induced increase in aortic cholesterol was reduced significantly by naltrexone administration, although aortic cholesterol levels in this group remained significantly higher than those of the control group (24% increase). Chronic treatment with naltrexone in the manner utilized here was not associated with overt signs of withdrawal in the morphine-drinking mice.

Total morphine intake over the 28-day period was 112 ± 2.5 mg. Mean total morphine intake in the group that also received naltrexone was lower (101 ± 5.9 mg), but this difference was not significant. Morphine treatment also did not affect the rate of body weight gain (data not shown).

The effects of a second opiate agonist, loperamide (0.1% in the drinking water), were also examined. Loperamide, a relatively peripherally restricted agonist, did not affect cholesterol levels in the plasma, liver or aorta (Fig. 2). Total intake of loperamide was similar to that of morphine-drinking mice.

DISCUSSION

The relationship of elevated circulating cholesterol levels to coronary heart disease has been well documented [23], and the elevation of plasma cholesterol associated with stressful situations may contribute to the development of vascular disorders. In this report we suggest that endogenous opioids may play a role in immobilization stress-induced aberrations of cholesterol homeostasis, since these effects of stress could be blocked by an opiate antagonist, naltrexone, and duplicated by an opiate agonist, morphine. Restraint stress has been reported to activate endogenous opioid systems and produce naloxone reversible analgesia [24], although non-opioid substances may also be involved in this phenomenon [25]. These findings parallel our observations of the effects of shorter term chronic stress or morphine regimens on circulating cholesterol in the rat [26] and offer additional information regarding elevation of cholesterol in the liver and aorta following more prolonged exposure in a mouse model.

The liver is the major site for removal of cholesterol in the form of low density lipoprotein (LDL) from the organism. Elevated cellular cholesterol reduces the uptake of extracellular cholesterol by reducing the synthesis of new LDL receptors [27]. Therefore, the elevation of liver cholesterol which we observed with chronic stress or morphine may have contributed to the elevation in plasma cholesterol by reducing hepatic clearance of cholesterol-laden LDL. Indeed, in preliminary investigations monitoring the relative abundance of the various lipoprotein subtypes in the plasma, we have noted a trend for elevated LDL with chronic immobilization stress or morphine treatment (unpublished observation). Opiates have not been studied very extensively with regard to their effects on cholesterol and lipid metabolism, although a secondary effect of respiratory depression induced by morphine is the inhibition of hepatic protein synthesis [28]. Since the primary control for cholesterol homeostasis is the lipoprotein receptor, impaired protein synthesis may result in reduced or defective production of LDL receptors or of the recognition apoprotein B of LDL which is critical for binding to the receptor [29]. Morphine administration has also been associated with the elevation of hepatic triglyceride synthesis [30], indicating one other possible effect of opioids at the hepatic level with might affect overall lipid metabolism.

The elevation of aortic cholesterol in association with the increase in plasma cholesterol does strengthen the hypothesized link between stress and atherosclerosis. Our finding that naltrexone only partially reduced the aortic cholesterol elevation in the morphine and stressed rats, yet completely prevented the plasma and liver cholesterol elevations, would tend to agree with the hypothesis that aortic tissue is one of the most sensitive extra-hepatic sites for cholesterol deposition [5]. This may be attributable to the fact that smooth muscle tends to have a greater concentration of LDL receptors than other tissue [31].

We have observed similar opiate effects on biliary cholesterol levels in female guinea pigs [32]. Chronic morphine treatment led to a dramatic increase in the cholesterol content of newly secreted bile. Further work in the mouse has demonstrated the ability of chronic opiate treatment and stress to induce cholesterol gallstone development [17, 33]. This is of interest since human populations that have a high incidence of heart disease also tend to have a high incidence of gallstones [18]. Since endogenous opioids and morphine are known to elevate food

intake acutely [10, 11], an increase in intake of the high cholesterol diet could lead to the observed elevations in plasma cholesterol. However, we observed no differences in 24-hr food intake or in the rate of body weight gain.

Loperamide is an opiate agonist which is thought to be restricted to peripheral tissues when given in moderate doses [34]. The loperamide regimen we used in this study is as effective as morphine in facilitating gallstone formation in cholesterol fed mice [17]. However, loperamide was unlike morphine in that it did not raise cholesterol levels in the plasma, liver or aorta. Loperamide is four to twelve times more potent than morphine in blocking diarrhea, in displacing naloxone binding of brain homogenates [35], and in stimulating food intake [36]. Loperamide is similar to morphine in that it is capable of binding to both mu- and delta-opiate receptors [37]. Assuming that loperamide is more potent at the pertinent opiate receptor, the ineffectiveness of this peripherally restricted opiate agonist may indicate that the opioid receptors that are involved in stress-induced effects on cholesterol metabolism are centrally located. This then would also imply a central site for naltrexone in antagonizing stress-induced elevations in cholesterol levels. A central site appears feasible, as lateral hypothalamic stimulation can raise plasma cholesterol levels [38] and even induce atherosclerotic changes in the aorta [6].

In conclusion, activation of central endogenous opioid systems appears to produce changes in cholesterol transport which may eventually contribute to the pathogenesis of atherosclerosis. Further pharmacological and biochemical studies assessing a possible link between stress and coronary heart disease are warranted.

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