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Piroxicam modifies the effects of ethanol on isolated rat hepatocytes

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Abstract

It has been reported that piroxicam prevents the hepatic increase of triacylglycerides and thiobarbituric acid-reactive substances observed after acute ethanol intoxication in rats and also causes a decrease in blood ethanol concentration. The aim of this study was to assess the effect of piroxicam on these 3 metabolic indicators, using isolated rat hepatocytes incubated with ethanol or lactate, supplemented or not with epinephrine. Epinephrine stimulated the consumption of lactate, but not of ethanol. In the isolated hepatocytes, and in a dose-dependent fashion, piroxicam alone raised the consumption of lactate and ethanol, increased the triacylglyceride pool in cells incubated with lactate or ethanol, and decreased the content of thiobarbituric acid-reactive substances in cells incubated with ethanol, but not with lactate. Epinephrine blocked these actions of piroxicam, except the lowering of the content of thiobarbituric acid-reactive substances. Thus, piroxicam helps to control the oxidative stress produced in isolated hepatocytes by ethanol.

Keywords: Epinephrine; Ethanol; Isolated hepatocyte; Piroxicam; Thiobarbituric acid-reactive substance; Triacylglyceride

1. Introduction

Ethanol is the most extensively toxic compound consumed by humans (Williams et al., 1988; Multi-authorial, 1990). Ninety percent of people drink alcohol, 40–50% of men have temporary ethanol-induced problems, and 10% of men and 3–5% of women develop pervasive ethanol-related problems (alcoholism). Even light drinking may adversely interact with other medications and temporary heavier drinking can exacerbate most medical illnesses (Schuckit and Irwin, 1988). Acute ethanol intoxication is an unresolved therapeutic episode: means to ameliorate the temporary toxic actions of high doses of ethanol are absent in the medical armamentarium. Thus compounds with a potential protective effect against the acute metabolic actions of ethanol deserve additional study. Previous data from our laboratory (Zentella de Piña et al., 1992, 1993) have shown that some nonsteroidal anti-inflammatory drugs partially prevent the increase in liver triacylglycerides and thiobarbituric acid-reactive substances, the latter measured as an indication of cellular oxidative stress (Bondy and Pearson, 1993). Both substances are produced during acute intoxication of fasted rats with ethanol. In addition, one of the anti-inflammatory drugs tested, namely piroxicam, consistently produced a decrease in the levels of blood ethanol in comparison with those of control animals (Zentella de Piña et al., 1992). The aim of this work was to study the effect of piroxicam on the 3 studied indicators – triacylglycerides, thiobarbituric acid-reactive substances and ethanol consumption – in a simpler biological system, such as isolated rat liver cells, in order to facilitate the study of the actions of these nonsteroidal anti-inflammatory drugs, compounds which are extensively used in clinical medicine (Insel, 1990).

Ethanol was replaced by lactate in the control experiments, and the effect of piroxicam on the 3 above-mentioned indicators was also recorded. Epinephrine was routinely included in the assays since ethanol, in the doses used in the in vivo experiments reported before (Zentella de Piña et al., 1992), increases the concentration of epinephrine (Brodie and Maikel, 1963). Additionally, in a completely different system, nonsteroidal anti-inflammatory drugs antagonized the antihypertensive effect of β-adrenoceptor antagonists (Johnson et al., 1994). Thus, we explored whether some of the beneficial actions of piroxicam in ethanol intoxication might be a consequence of a

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combined action of epinephrine and the anti-inflammatory compounds.

2. Materials and methods

Male Wistar rats (200–250 g) were fed ad libitum on a commercial diet (Nutricubos from México) supplemented with minerals and vitamins in order to achieve an optimal growth rate. Animals were fasted 48 h before treatment, but free access to water was permitted. Isolated hepatocytes were prepared by the method of Berry and Friend (1969) as detailed previously (Guinzberg et al., 1987). Cell viability was assayed by the trypan blue exclusion method; experiments were performed when 90% viability was recorded. The isolated cells were incubated as described in the figure legends. After incubation, triacylglyceride content, thiobarbituric acid-reactive substances production, lactate and ethanol consumption and protein concentration were quantified. Each assay was routinely performed in duplicate.

Triacylglycerides were extracted with heptane and hydrolyzed with KOH as described (Gottfried and Rosenberg, 1973). Glycerol was then oxidized with periodate and the resulting formaldehyde condensed with acetylacetone to form a yellow dehydrolutidine derivative, which was measured colorimetrically. Lipid peroxidation was quantified by the thiobarbituric acid-reactive substances method previously described in detail (Zentella de Piña et al., 1993). The concentration of thiobarbituric acid-reactive substances in the samples can be calculated using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} (Wills, 1969). For ethanol or lactate quantification, 50-μl aliquots of the complete incubation mixture, including hepatocytes, were placed in 400 μl of ice-cold 0.33 M perchloric acid, at zero time and after 1 h of incubation. Tubes were stirred and then centrifuged; samples of the supernatants were used to measure either ethanol or lactate. Ethanol was quantified by the Bernt and Gutmann technique (Bernt and Gutmann, 1974): the amount of ethanol, in the presence of an excess of NAD^+ and alcohol dehydrogenase (alcohol:NAD oxidoreductase, EC 1.1.1.1), is proportional to the amount of NADH formed, wich was measured spectrophotometrically. Lactate was determined by the Gutmann and Wahlefeld method (Gutmann and Wahlefeld, 1974) according to the same principle but replacing alcohol dehydrogenase by lactate dehydrogenase (L-lactate:NAD oxidoreductase, EC 1.1.1.27). Protein was determined as described (Bradford, 1976), using bovine albumin as standard. Statistical analyses were made by analysis of variance (Zar, 1984a) for each piroxicam concentration, followed by pairwise comparisons by Tukey (Zar, 1984b) or by Fisher (Levy, 1976). Data recorded in control cells vs. those obtained in cells treated with each concentration of piroxicam were compared by using the Student *t*-test. A difference was considered significant when $P < 0.05$.

3. Results

The consumption of lactate or ethanol by the isolated hepatocytes is presented in Fig. 1. As expected, in the absence of piroxicam, epinephrine stimulated the utilization of lactate ($P < 0.05$) but not ethanol in hepatocyte cells obtained from fasted rats. Piroxicam alone increased, in a dose-dependent fashion, the consumption of lactate, this increase being significant when the concentration of the anti-inflammatory drug reached 10^{-6} M ($P < 0.05$) and 10^{-5} M ($P < 0.01$), compared to the values recorded without piroxicam. Simultaneous addition of epinephrine and piroxicam resulted in an apparent mutual antagonism of the effects of the compounds. Individual values varied widely and the effect was not statistically significant. Similarly, piroxicam alone increased ethanol consumption; the response was dose-dependent from 10^{-9} M to 10^{-6} M ($P < 0.05$ for 10^{-6} M). The actions of the anti-inflammatory drug on ethanol utilization were the same in hepatocytes incubated in the presence of epinephrine, but the increase was not statistically significant (Fig. 1).

The content of triacylglycerides at the end of the 60-min incubation was similar in hepatocytes incubated with lactate or ethanol, with or without epinephrine (Fig. 2). Independent of the presence or absence of epinephrine, piroxicam produced a dose-dependent rise in the pool of triacylglycerides: the difference between the triacylglyceride content of cells incubated with lactate without piroxicam vs. that of cells incubated with 10^{-5} or 10^{-4} M piroxicam was significant at $P < 0.05$; for cells incubated with ethanol without piroxicam and those incubated with 10^{-6} M piroxicam the difference was significant at $P < 0.05$, with 10^{-5} M piroxicam the difference was significant ($P = 0.021$), and with 10^{-4} piroxicam the difference was significant also ($P = 0.014$). The differences in triacylglyceride content were significant by analysis of vari-

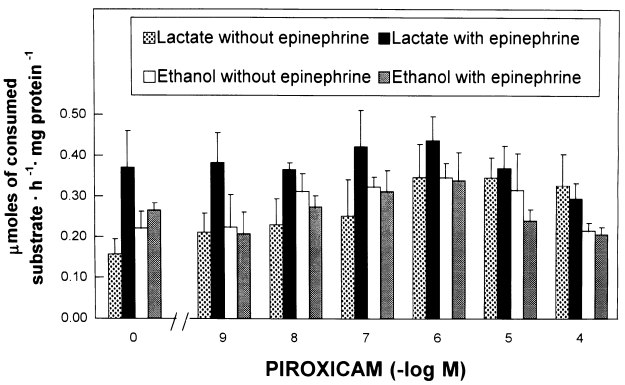

Fig. 1. Effect of piroxicam, in the absence or in the presence of 1×10^{-6} M epinephrine, on the consumption of lactate or ethanol by isolated hepatocytes. Isolated rat liver cells, equivalent to 10–15 mg of protein, were incubated under continuous shaking in Krebs-Ringer bicarbonate buffer, supplemented with 50 mM sodium lactate pH 7.4 or 50 mM ethanol, in an atmosphere of O_2 /CO_2 (19:1), for 60 min, at 37°C. Each bar represents the average ± standard error with $n = 4$–9.

ance test at 10^{-5} M piroxicam. Pairwise comparisons by Fisher's least-significant difference test indicated that with 10^{-5} M piroxicam the difference in triacylglyceride content between cells incubated with ethanol without epinephrine vs. with ethanol and with epinephrine was just significant ($P = 0.049$) and the difference between cells incubated with ethanol without epinephrine vs. those incubated with lactate with epinephrine was significant at $P = 0.017$ (Fig. 2). The combination of epinephrine and piroxicam produced a moderate stimulation, which was not significant, in the triacylglycerides pool of hepatocytes incubated with lactate. This stimulation was not observed in the cells incubated with ethanol.

Without piroxicam, but in the presence of epinephrine, the isolated liver cells had lower values of thiobarbituric acid-reactive substances when incubated with ethanol instead of with lactate ($P < 0.05$, Fig. 3). No significant differences were observed among the other 3 groups of cells incubated in the absence of piroxicam, namely, lactate or ethanol without epinephrine, and lactate with epinephrine. The anti-inflammatory compounds show a tendency to decrease the amount of thiobarbituric acid-reactive substances in liver cells incubated with lactate but without epinephrine. The piroxicam-induced lowering of the content of thiobarbituric acid-reactive substances in cells was more evident when ethanol was the substrate ($P < 0.05$ comparing control values vs. 10^{-4} piroxicam) and was observed even in the presence of epinephrine (Fig. 3).

Significant differences in the amount of thiobarbituric acid-reactive substances were found by analysis of variance for 10^{-6}, 10^{-5} and 10^{-4} M piroxicam. Tukey's pairwise comparison for 10^{-6} M piroxicam indicated differences in thiobarbituric acid-reactive substances between cells incubated with ethanol with epinephrine and those incubated with lactate with epinephrine ($P = 0.033$); for 10^{-5} M piroxicam, the difference between cells incubated with ethanol without epinephrine and cells incubated with lactate with epinephrine was significant at $P = 0.033$, and

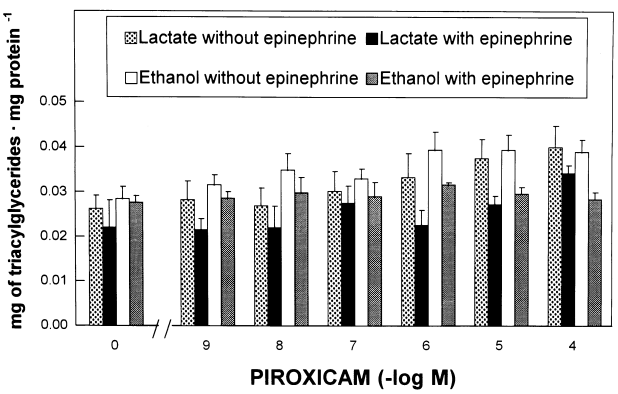

Fig. 2. Effect of piroxicam, in the absence or in the presence of 1×10^{-6} M epinephrine, on the pool of triacylglycerides in isolated hepatocytes incubated with lactate or ethanol. Experimental conditions and symbols are as described in Fig. 1.

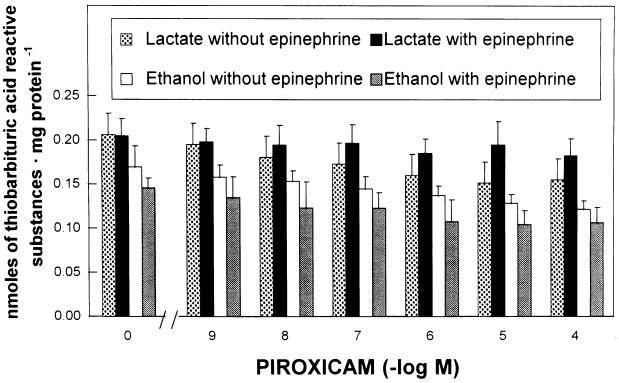

Fig. 3. Effect of piroxicam, in the absence or in the presence of 1×10^{-6} M epinephrine, on the content of thiobarbituric acid-reactive substances in isolated hepatocytes incubated with lactate or ethanol. Experimental conditions and symbols are as described in Fig. 1.

for cells incubated with ethanol with epinephrine vs. cells incubated with lactate with epinephrine the difference was significant at $P = 0.011$; finally for 10^{-4} M piroxicam, cells incubated with ethanol without epinephrine vs. cells incubated with lactate with epinephrine the difference was significant at $P = 0.029$, and for cells incubated with ethanol with epinephrine vs. cells incubated with lactate with epinephrine the difference was significant at $P = 0.016$.

4. Discussion

This paper describes attempts to characterize the actions of a nonsteroidal anti-inflammatory drug that counteracts some of the acute effects of ethanol in the rat. With this aim isolated hepatocytes in which lactate consumption was increased by epinephrine (Fig. 1) were adopted as a simpler and more reliable model than the whole animal. Therefore, previous results obtained with piroxicam treatment of ethanol-intoxicated rats (Zentella de Piña et al., 1992) were compared with the data recorded in this work with isolated cells incubated with ethanol and increasing doses of the anti-inflammatory compound.

In rats acutely intoxicated with ethanol, piroxicam consistently produces a decrease in the blood concentration of ethanol in comparison with control animals without piroxicam. On that basis, it was suggested that piroxicam stimulates hepatic ethanol oxidation (Zentella de Piña et al., 1992). The increase in ethanol consumption observed in hepatocytes incubated without epinephrine (Fig. 1), but with doses of piroxicam considered in the therapeutic range (Insel, 1990), is compatible with the previous suggestion. Interestingly, piroxicam alone stimulated also, and to a greater extent, the consumption of lactate by hepatic cells from fasted rats (Fig. 1). Studies designed to elucidate whether the effect of piroxicam on lactate consumption includes a stimulation of its oxidation or of its conversion to some storage molecule (glycogen or triacylglyc-

erides) are required. In regard to the piroxicam-mediated stimulation of substrate oxidation, an alternative that should be experimentally considered includes activation of the NADH shuttle, leading to recycling of NAD^+ and thereby overcoming the limiting step in their hepatic oxidation (Meijer et al., 1975; Brand and Murphy, 1987, for a general review).

The nonsteroidal anti-inflammatory drug prevents the hepatic increase in triacylglycerides resulting from acute intoxication of rats with ethanol (Zentella de Piña et al., 1992). In the isolated hepatocytes incubated without epinephrine, piroxicam produced opposite results: a dose-dependent significant increase in the pool of triacylglycerides (Fig. 2). It is of interest that such an increase was directly proportional to the amount of substrate consumed (with a correlation coefficient, $r = 0.85$), independent of whether the substrate was lactate or ethanol, but only in the absence of epinephrine. In any event, the in vitro results (Fig. 2) are opposite to the in vivo ones (Zentella de Piña et al., 1992). In this respect it could be concluded that the action of piroxicam in the whole rat, whereby the ethanol-mediated accumulation of hepatic triacylglycerides is avoided, is related to the mobilization of storage lipids from adipose tissue. There are reports that acute ethanol administration activates the liberation of free fatty acid from adipose tissue (Di Luzio, 1963) and that a different nonsteroidal anti-inflammatory drug, acetyl salicylic acid, inhibits this mobilization (Carlson et al., 1965). The interference of epinephrine in the action of piroxicam on triacylglycerides in isolated hepatocytes (Fig. 2) deserves further study.

In rats acutely intoxicated with ethanol, piroxicam can lessen the hepatic increase in malondialdehyde produced by the hepatotoxic compound (Zentella de Piña et al., 1992). In a similar fashion, the anti-inflammatory drug decreased the content of malondialdehyde, here reported as thiobarbituric acid-reactive substances (Fig. 3), in hepatocytes incubated with lactate or ethanol. Assuming that thiobarbituric acid-reactive substances are indicative of cellular oxidative stress, the results in Fig. 3 are consistent with in vitro studies showing an inhibitory action of piroxicam on the release of superoxide radicals from activated neutrophils (Kaplan et al., 1984) and from isolated Kupffer cells in primary cultures (Miranda et al., 1995). Although the underlying mechanism for this protective action of piroxicam remains unknown, it is of interest that piroxicam, both in vivo and in vitro, and other nonsteroidal anti-inflammatory drugs in vivo (Zentella de Piña et al., 1993) share the action of preventing the hepatic increase in thiobarbituric acid-reactive substances resulting from the acute exposure to ethanol. This information could be important due to the widespread clinical use of nonsteroidal anti-inflammatory drugs (Insel, 1990).

In the in vivo experiments, piroxicam prevented the hepatic increase in triacylglycerides and thiobarbituric acid-reactive substances elicited by alcohol intoxication. It was then considered that a smaller pool of triacylglycerides might result in a lower production of thiobarbituric acid-reactive substances (Zentella de Piña et al., 1992). Moreover, in the in vitro studies piroxicam alone produced an inverse relationship between triacylglycerides (Fig. 2), which increased, and thiobarbituric acid-reactive substances (Fig. 3), which decreased. Therefore, these indicators of the response to piroxicam are independent, at least at the level of isolated liver cells.

Our data are insufficient to document an epinephrine-piroxicam interaction, like the one analyzed between nonsteroidal anti-inflammatory drugs and β-adrenoceptor antagonist (Johnson et al., 1994). In general, the actions of piroxicam were best observed in the absence of added epinephrine.

In conclusion, in isolated rat liver cells piroxicam contributes to controlling the oxidative stress produced by incubation with ethanol. This simple model for studying the action of piroxicam is suitable for use in further studies.

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