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Non-steroidal anti-inflammatory drugs inhibit epinephrine- and cAMP-mediated lipolysis in isolated rat adipocytes

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Abstract

Acute ethanol intoxication increased triacylglycerides (TAG) and thiobarbituric acid reactive substances (TBARS) in liver and promoted the liberation of epinephrine. Four non-steroidal anti-inflammatory drugs (NSAIDs) – aspirin, naproxen, nimesulide and piroxicam – prevented this increase in TAG and TBARS. Because fatty acids provided by adipose tissue contribute substantially to elevated hepatic TAG in ethanol-intoxicated rats, it was thought that the NSAIDs might reduce epinephrine-stimulated lipolysis in these rats. Isolated rat adipocytes were activated with epinephrine in the presence or absence of the NSAIDs. The NSAIDs inhibited epinephrine-stimulated lipolysis. These drugs did not modify the binding of dihydroalprenolol (β -adrenergic agonist) to their receptors in isolated guinea-pig liver membranes. The NSAIDs, at concentrations 3000-fold lower than that of cAMP, inhibited stimulated lipolysis by this messenger. In conclusion, aspirin, naproxen, nimesulide and piroxicam reduce the release of fatty acids from adipose tissue to the liver by inhibiting the epinephrine-stimulated lipolysis, and this, in part, explains the protective action of these NSAIDs against hepatic signs of acute ethanol intoxication.

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) exert their anti-inflammatory, analgesic and antipyretic actions, at least in part, by inhibition of the enzyme cyclooxygenase (Vane 1971). More recently, other pharmacological activities of these compounds have been described. Yin et al (1998) reported that aspirin and salicylate inhibit in-vitro and in-vivo the activity of I κ B kinase- β , thus impairing the release and mobilization of NF- κ B, a key cellular regulator of the inflammatory response. In an in-vivo model of acute inflammation, Cronstein et al (1999) demonstrated that aspirin and salicylate worked by promoting the release of the anti-inflammatory autacoid adenosine. Many of the effects of NSAIDs that are not mediated by cyclooxygenase are thought to be owing to interference with some step of the extensively distributed signal transduction systems associated with cell membranes (Riveros-Rosas et al 1999). Thus, it might be expected that NSAIDs would antagonize some hormone-induced and neurotransmitter-induced responses. An antagonism between catecholamines and NSAIDs has been documented for in-vivo experiments (Johnson et al 1994) and for the metabolic responses of epinephrine and piroxicam in isolated rat hepatocytes (Riveros-Rosas et al 1999).

In this work, the ability of four NSAIDs – aspirin, naproxen, nimesulide and piroxicam – to inhibit epinephrine-stimulated lipolysis in isolated rat adipocytes was assessed in an attempt to explain the observation that these compounds prevented the hepatic increase of triacylglycerides (TAG) and thiobarbituric acid reactive substances (TBARS) in rats administered high doses of ethanol (Zentella de Piña et al 1992, 1993). The four NSAIDs chosen are extensively used in humans. In particular, aspirin was chosen because it is the standard model with which to compare and evaluate other NSAIDs. Some advantage or difference with reference to aspirin was sought for the remaining selected NSAIDs; naproxen is better tolerated, nimesulide acts preferentially on cyclooxygenase 2, and piroxicam has a longer half-life (Insel 1990).

An excessive mobilization of lipids from the adipose tissue was claimed to be the main cause of the resulting fatty liver after acute ethanol ingestion (Horning et al 1960), and ethanol in large doses increases the release of catecholamines and other lipolytic hormones (Vogel et al 1986). If the NSAIDs interfere with the stimulatory action of epinephrine in isolated adipocytes, this effect might help to explain the results of our previous studies (Zentella de Piña et al 1992, 1993). A single report in the literature deals with this topic: Stone et al (1969) demonstrated that 5×10^{-3} M sodium salicylate reduced the rate of lipolysis in isolated rat adipocytes incubated with 5×10^{-7} M norepinephrine or 0.1 mM dibutyryl cyclic AMP plus 10^{-5} M theophylline, or 1.0 mM dibutyryl cyclic AMP plus 4×10^{-3} M theophylline.

Materials and Methods

Animals

Male Wistar rats, 200–240 g were fed *ad libitum* with a commercial diet (Nutricubos, Purina, México). They were fasted overnight, but had free access to water. Rats were decapitated and the epididymal fat pads from two rats were immediately removed. The Ethics Committee of the School of Medicine at the National University of México approved the experiments in accordance with the International Animal Care and Use Committee.

Chemicals and reagents

ATP, (\pm) alprenolol, NAD, glycerol, thiobarbituric acid, epinephrine, aspirin, naproxen, nimesulide, piroxicam, dibutyryl cAMP, glycerol-3-phosphate oxidase, glycerokinase, Triton X-100, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 4-chlorophenol,

4-aminoantipirine, peroxidase, and albumin were all obtained from Sigma (St Louis, MO, USA); *l*-[*propyl*-2,3- 3 H]dihydroalprenolol (120 Ci mmol^{-1}) was supplied by New England Nuclear Co (USA); EDTA, ethanol, glucose and magnesium sulfate were obtained from Baker (Edo de México, México).

Lipolysis experiments

Isolated adipocytes were obtained by the method of Rodbell (1964) as modified by Honnor et al (1985) with the main purpose of obtaining cells with low basal levels of cAMP. In brief, fat pads from two rats were cut up with fine scissors. Digestion was performed with 1 g of minced pad in 10 mL collagenase (1 mg mL^{-1}) for 30 min, with vigorous shaking, in 1% defatted bovine serum albumin (BSA; fraction v) in Ringer Krebs media, pH 7.4. The Ringer Krebs was enriched with 25 mM HEPES adjusted to pH 7.4, 2.5 mM CaCl_2 , 2 mM glucose and 200 nM adenosine. Cells were filtered through nylon cloth. After three cycles of centrifugation (1 min each) at 220 g, elimination of the solution lying below the fat cake, and resuspension in 1% BSA in Ringer Krebs enriched solution, the fat cake was weighed; 1.0 g of this wet weight of cells were suspended in 1.25 mL Ringer Krebs enriched solution in which the concentration of BSA was raised to 4% instead of 1%. Lipolysis was assayed in 100- μL aliquots of this suspension added to 700 μL of incubation medium, pre-warmed to 37°C , which consisted of 4% BSA in Ringer Krebs enriched solution plus epinephrine or dibutyryl cAMP with or without each of the selected NSAIDs. Incubation was performed at 37°C for 30 min. The adipocytes were dispersed during incubation by shaking at 160 cycles min^{-1} . Lipolysis was stopped by transferring the tubes from 37°C to an ice bath after 5 min. The tubes were then centrifuged at 10000 g at 4°C for 10 min. An aliquot of 100 μL from the solution lying below the fat cake was carefully transferred to a new tube to measure liberated glycerol. Glycerol was estimated by a colorimetric method recommended by Warnick (1986). In this method, glycerol is converted to glycerol 1-phosphate in the presence of glycerol kinase and ATP; glycerol 1-phosphate was oxidized with the aid of glycerol 3-phosphate oxidase to produce equimolecular amounts of H_2O_2 and dihydroxyacetone phosphate. H_2O_2 was reacted with phenol, aminoantipirine and peroxidase enzyme to give a quinoneimine dye proportional to the content of the H_2O_2 present.

All NSAIDs were assayed at a concentration of 3×10^{-6} M. This concentration was recovered in plasma of individuals receiving a single therapeutic dose of

piroxicam (Insel 1990). On an equimolecular basis, piroxicam is the most potent of the four NSAIDs used in this study (Insel 1990).

Membrane preparation

Liver plasma membranes were prepared from male guinea-pigs (450–500 g) after homogenization in a hypotonic solution (1 mM NaHCO_3) with a Dounce glass device (four strokes), according to the procedure, up to step 11, devised by Neville (1968). Membranes were resuspended in 50 mM Tris-HCl, pH 7.4, containing 10 mM MgCl_2 and stored in liquid nitrogen at -70°C until use (within 2 weeks); protein was measured by the method of Bradford (1976).

Saturation-binding and competence experiments

Saturation-binding assays for β -adrenoreceptors were performed in triplicate with 100- μg samples of membrane protein. Membranes were incubated in 50 mM Tris-HCl, pH 7.4, containing 10 mM MgCl_2 with eight increasing concentrations of [^3H]dihydroalprenolol (0.1–30 nM). Non-specific binding was obtained in the presence of 10 μM cold alprenolol in a final volume of 0.25 mL at 37°C for 10 min (Reyes-Salcido & Villalobos-Molina 1999). The same protocol was followed in tubes supplemented with 50 mM ethanol. Samples were filtered through a Brandell cell harvester (Gaithersburg, MD, USA) over Whatman GF/C filters with three washes of 5 mL ice-cold binding buffer. After drying the filters, 5 mL Triton-xylene scintillation liquid was added and radioactivity in the filters was determined in a liquid scintillation spectrometer (Beckman LS6000SC) at 63% efficiency. Specific binding was 50–55% at the K_d for [^3H]dihydroalprenolol.

For competence experiments, binding for [^3H]dihydroalprenolol (at the concentration of the calculated K_d) was challenged to compete with each of the NSAIDs and incubated in triplicate at 14 different concentrations ranging from 3×10^{-8} to 3×10^{-2} M. Each complete experiment was repeated three times, either in the absence or presence of 50 mM ethanol.

Calculations and statistical analysis

Each value in the figures is the mean of six independent experiments in which adipocytes were stimulated with epinephrine or dibutyryl cAMP, and three independent experiments in which adipocytes were stimulated with epinephrine or dibutyryl cAMP supplemented with one of the NSAIDs. The glycerol values are expressed as

mean \pm s.d. Differences in dose–response curves between the stimulated cells with epinephrine or dibutyryl cAMP and the stimulated cells plus one of the NSAIDs were analysed by one-way analysis of variance followed by Dunn's multiple comparison test recommended for unpaired samples (Sigma Stat for Windows, version 1.0; Jandel Corporation, San Rafael, CA, USA). In all analyses, $P < 0.05$ was taken as the level of significance. The concentration and affinity of β -adrenoreceptors were determined in three and eight separate experiments by saturation binding and Scatchard analysis using the non-linear curve-fitting EBDA-LIGAND program (Biosoft, Elsevier, Cambridge, UK). Student's t -test for unpaired observations was used to compare the B_{max} and affinity between groups.

Results

The dose–response curve for epinephrine-stimulated lipolysis is shown in Figure 1. High doses of the hormone were used to show the wide physiological reserve

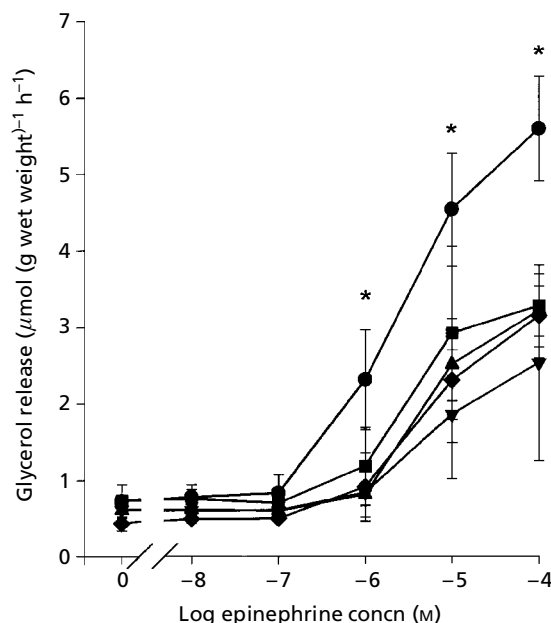


Figure 1 Effect of different concentrations of NSAIDs on epinephrine-stimulated glycerol release in isolated rat adipocytes. Adipocytes were incubated with different concentrations of epinephrine alone (●) or in the presence of 3×10^{-6} M aspirin (■), naproxen (◆), nimesulide (▲) or piroxicam (▼). All samples were incubated for 30 min at 37°C . Each value represents the mean \pm s.d. for six individual assays in adipocytes incubated with epinephrine and three individual assays in adipocytes incubated with epinephrine plus one of the NSAIDs. * $P < 0.05$, epinephrine-treated cells vs epinephrine plus one of the NSAIDs.

Table 1 Receptor number (B_{\max}) and K_d for [3 H]dihydroalprenolol in isolated hepatocyte membranes.

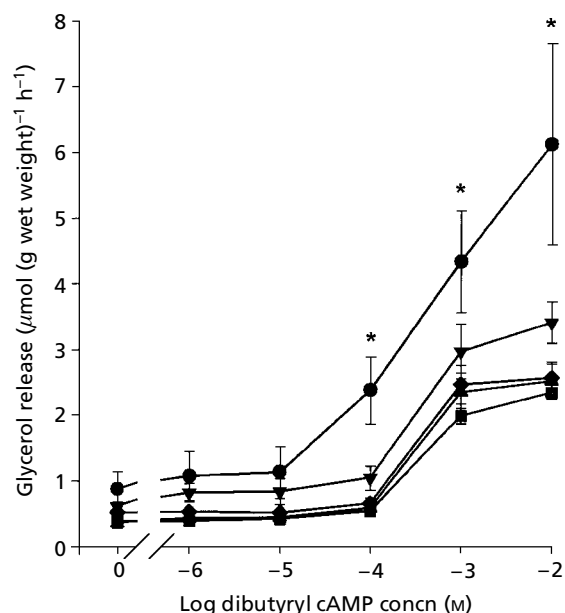
Incubation medium	B_{\max} (fmol (mg protein) $^{-1}$)	K_d (nM)
In the absence of ethanol	2167 \pm 539 (n = 8)	8.9 \pm 1.6 (n = 8)
With 50 mM ethanol	1840 \pm 435 (n = 3)	23.6 \pm 2.8 (n = 3)

Values are mean \pm s.e.

response of the prepared adipocytes and also to test whether the pharmacological doses of the NSAIDs could act at physiological as well as supra-physiological doses of epinephrine. The four NSAIDs used in this study exhibited a clear inhibitory action on epinephrine-stimulated lipolysis. The inhibitory action of the NSAIDs recorded in the absence of epinephrine was not statistically significant (Figure 1). In epinephrine-stimulated lipolysis, the inhibitory action of the NSAIDs was statistically significant and was maintained even when the concentration of each NSAID was 33-times lower than the concentration of the stimulatory hormone (Figure 1). The results showed a trend of potency in this assay in the following order: piroxicam (the most potent) > naproxen > nimesulide > aspirin. However, differences among NSAIDs were not statistically significant.

The main epinephrine receptor localized in rat adipocytes is of the β subtype. Therefore, the ability of ethanol and the selected NSAIDs to modulate the binding to this receptor of dihydroalprenolol, a model β -adrenergic agonist, was studied. Displacement curves for [3 H]dihydroalprenolol (18.9 nM, K_d in Table 1) to β -adrenergic receptors were established for aspirin, naproxen and piroxicam. Under the experimental conditions studied here, the presence of alcohol had no statistically significant effect on the binding of dihydroalprenolol to the receptor, as evidenced by the similar B_{\max} and K_d values (Table 1). None of the NSAIDs tested had a statistically significant effect on the binding of dihydroalprenolol to the receptor.

The next step was to assay the capacity of NSAIDs to modify the stimulatory action of cAMP, the second messenger involved in the process of β -receptor stimulation of lipolysis. Dibutyl cAMP increased glycerol release in a dose-dependent manner, as previously documented (Figure 2). The NSAIDs tested here reduced this stimulatory action, although the concentration of the anti-inflammatory agents was maintained at 33–3333 times lower than the concentration of cAMP (Figure 2).

**Figure 2** Effect of different concentrations of NSAIDs on dibutyl cAMP-stimulated glycerol release in isolated rat adipocytes. Adipocytes were incubated with different concentrations of dibutyl cAMP alone (●) or in the presence of 3×10^{-6} M aspirin (■), naproxen (◆), nimesulide (▲) or piroxicam (▼). All samples were incubated for 30 min at 37°C. Each value represents the mean \pm s.d. for six individual assays in adipocytes incubated with dibutyl cAMP and three individual assays in adipocytes incubated with dibutyl cAMP plus one of the NSAIDs. * $P < 0.05$, cAMP-stimulated cells vs cAMP plus one of the NSAIDs.

The results showed a trend of potency in this assay in the following order: aspirin (the most potent) > nimesulide > naproxen > piroxicam (Figure 2). However, differences among NSAIDs were not statistically significant. Interestingly, this trend was the exact opposite of that observed with the lipolysis assay in Figure 1.

Discussion

This work demonstrates that the stimulatory action of epinephrine on epididymal adipose tissue mediated by cAMP was diminished by the presence of four different NSAIDs at concentrations considered below their therapeutic range. This inhibitory action should be added to the growing list of biological effects exhibited by NSAIDs; to date, no connection between cyclooxygenase activation and catecholamine signalling pathway has been reported.

The results help to explain some effects of NSAIDs that limit the metabolic disturbances produced in the liver after acute intoxication with ethanol. Admini-

stration of ethanol at doses of 5 g kg⁻¹ bodyweight leads to an increase in the hepatic content of TAG and TBARS (Zentella de Piña et al 1992, 1993), and to a 6- to 20-fold greater secretion of epinephrine (Vogel et al 1986), a hormone with marked lipolytic action. An increased mobilization of fatty acids from the storage tissues is an important contribution to the fatty liver observed after ethanol intoxication (Horning et al 1960). On the other hand, pharmacological doses of NSAIDs (Insel 1990) partially reversed the hepatic increase in TAG and TBARS promoted by ethanol intoxication (Zentella de Piña et al 1992, 1993), and, to date, no explanation has been offered for this action. The inhibitory effect of NSAIDs on the epinephrine-mediated lipolysis described here can provide an experimental framework with which to explain the beneficial actions of some NSAIDs against the deleterious effects of ethanol on the liver. Thus, in animals intoxicated with high doses of ethanol, NSAIDs diminished the stimulatory effect of an elevated concentration of epinephrine, decreasing the release of fatty acids from the epididymal fat pad cells to the liver and maintaining the hepatic pool of TAG at near normal values. Interference with the hepatic availability of fatty acids for peroxidation reactions might be related to the marked reduction of TBARS in the liver of ethanol-intoxicated rats treated with NSAIDs (Zentella de Piña et al 1992, 1993).

The metabolic antagonism between epinephrine and NSAIDs is not limited to lipolysis in adipocytes; in isolated rat hepatocytes, piroxicam blocked epinephrine-mediated glucose release from lactate (Riveros-Rosas et al 1999). The lack of effect of the different NSAIDs on dihydroalprenolol binding to the epinephrine receptors on guinea-pig cell membranes, together with the reduced dibutyl cAMP-dependent lipolysis of rat adipocytes, suggests that NSAIDs interfere with the epinephrine pathway at or downstream of the cAMP-dependent protein kinase.

Ethanol and NSAIDs are among the most consumed drugs by humans. This work highlights the need to be aware of, and to extend the information available on, the interplay between simultaneous presence of acute ethanol intoxication and the pharmacological use of NSAIDs. Results of this study are limited to the acute use of both compounds and the potential antagonism of these drugs at the level of the adrenergic hormones acting on lipolysis. In this particular case, the antagonism appears profitable to the liver, impairing some toxic effects of ethanol alone by limiting the availability of fatty acids provided by adipocytes. Nevertheless, chronic use or abuse of both compounds might offer a different picture in which the interplay between these

molecules may be broader, and may include other cellular types apart from adipocytes and hepatocytes (Riveros-Rosas et al 1999) and additional regulatory systems such as control of genetic expression. Knowledge of the relationship between ethanol and NSAIDs is necessary in the clinical setting to better understand the possible gains and risks of chronic use of NSAIDs in chronic alcoholics.

Conclusions

The results of this work show that pharmacological doses of NSAIDs can affect the release of fatty acids from deposits by inhibiting the action of epinephrine on adipocytes. Furthermore, some metabolic disturbances observed in the liver of rats acutely intoxicated with ethanol can be prevented by limiting the availability of fatty acids to the liver by an inhibition of lipolysis.

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