Fenofibrate -a PPARα agonist- increases alcohol dehydrogenase levels in the liver: implications for its possible use as an ethanol-aversive drug

Fenofibrato -un agonista de PPARα- incrementa los niveles de la alcohol deshidrogenasa hepática: implicaciones para su posible uso como una droga aversiva al etanol

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Abstract

After ethanol consumption, disulfiram increases blood-acetaldehyde levels, generating an aversive reaction that deters alcohol drinking. Given the major secondary effects of disulfiram, finding other effective drugs to reduce alcohol consumption in individuals with alcohol-use-disorder is highly desirable. It has been reported that administering fenofibrate to high-drinking rats increases hepatic catalase levels and blood acetaldehyde after administering ethanol and a 60-70% inhibition of voluntary alcohol intake. This work evaluated whether fenofibrate has an additional effect on the activity of other ethanol-metabolizing enzymes, which could contribute to the high acetaldehyde levels generated upon administering ethanol. Male high-drinker rats were allowed to voluntary drink 10% ethanol or water for 2 months. Subsequently, fenofibrate (100 mg/kg/day) or vehicle was administered orally for 14 days. Then, alcohol dehydrogenase (ADH1) and aldehyde dehydrogenase (ALDH2) protein levels and enzymatic activities in the livers were quantified. Fenofibrate treatment produced a marked increase in ADH1 protein levels (396% ± 18%, $p < 0.001$) and enzymatic activity (425% ± 25%, $p < 0.001$). Fenofibrate did not result in differences in ALDH2 activity or in ALDH2 protein levels. The studies show that treatment with fenofibrate not only increased the activity of catalase in the liver of alcohol-drinking rats, as reported earlier, but also increased the levels and enzymatic activity of ADH1, while ALDH2 remained unchanged. The increases in ADH1 contribute to explaining the remarkable effect of fenofibrate in raising blood levels of acetaldehyde in ethanol-consuming animals, in which a marked reduction of alcohol intake is recorded.

Key Words: Fibrate; Peroxisome proliferator-activated receptor; PPAR; Alcohol dehydrogenase; Alcohol use disorder treatment.

Resumen

Tras consumir etanol, el disulfiram incrementa los niveles de acetaldehído en sangre y genera una reacción aversiva que desalienta el consumo de alcohol. Dados los importantes efectos secundarios del disulfiram, es altamente deseable hallar otros fármacos efectivos para tratar el trastorno por uso de alcohol. Se ha reportado que administrar fenofibrato a ratas altamente bebedoras de alcohol aumenta los niveles de catalasa hepática y acetaldehído en sangre después de la administración de etanol, y disminuye el consumo voluntario de alcohol (60-70%). Este trabajo evalúa si el fenofibrato tiene un efecto adicional sobre la actividad de otras enzimas en el metabolismo del etanol que podría contribuir a generar altos niveles de acetaldehído. Se permitió a ratas macho altamente bebedoras beber voluntariamente etanol 10% durante 2 meses. Después, se les administró oralmente fenofibrato (100 mg/kg/día) o solo vehículo durante 14 días. Tras eso, se midieron los niveles hepáticos y actividades enzimáticas de alcohol deshidrogenasa (ADH1) y de aldehído deshidrogenasa (ALDH2). El fenofibrato produjo un marcado aumento en los niveles proteicos de ADH1 (396% ± 18%, $p <$,001) y de actividad enzimática (425% ± 25%, $p <$,001) sin alterar los niveles protéicos ni la actividad de ALDH2. Los resultados muestran que el tratamiento con fenofibrato no solo aumenta la actividad de catalasa en el hígado de ratas bebedoras de alcohol, sino que también incrementa los niveles y la actividad de ADH1, sin alterar ALDH2. Esto contribuye a explicar el notable efecto del fenofibrato en aumentar los niveles de acetaldehído en sangre en animales bebedores de alcohol, en los que se registra una marcada reducción en la ingesta de etanol.

Palabras clave: Fibrato; Receptor activado por proliferadores de peroxisomas; PPAR; Alcohol deshidrogenasa; Tratamiento trastorno por uso de alcohol.

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There are three approved drugs for the treatment of alcohol use disorder: naltrexone, acamprosate and disulfiram. Despite their use worldwide, the efficacy of these drugs is quite limited (between 10-13%) (Maisel, Blodgett, Wilbourne, Humphreys & Finney, 2013; Skinner, Lahmek, Pham & Aubin, 2014). Disulfiram acts by inhibiting aldehyde dehydrogenase (ALDH2), which results in an increase of blood acetaldehyde levels when the individual drinks alcohol. The rapid accumulation of acetaldehyde produces dysphoric effects that deter further alcohol consumption. However, disulfiram has important undesired effects which greatly limit its clinical use (Dupuy et al., 1995; Mark et al., 2003). Moreover, disulfiram is ineffective in a significant percentage of patients to reduce levels of alcohol consumption (Christensen, Moller, Ronsted, Angelo & Johansson, 1991). A recent clinical trial showed that disulfiram is not significantly different from placebo in promoting abstinence (Yoshimura et al., 2014).

In an attempt to find better drugs that could replace disulfiram in the clinic, we have evaluated the effect of fenofibrate. Fenofibrate belongs to a family of synthetic molecules called fibrates (which includes clofibrate, gemfibrozil, ciprofibrate, bezafibrate and fenofibrate) which are agonists of the peroxisome proliferator-activated receptor alpha (PPARα) (Gervois, Fruchart & Staels, 2007). Activation of PPARα in the liver leads to increased peroxisomal activity in this organ, which causes a higher rate of fatty acid oxidation. Based on this effect, fibrates are widely used in the clinic to treat elevated blood-triglyceride disorders (Gervois et al., 2007). Another consequence of this elevated peroxisomal activity is the increase of catalase levels in the liver (Karahanian, Quintanilla, Fernandez & Israel, 2014; Rivera-Meza et al., 2017). Like alcohol dehydrogenase (ADH1) and cytochrome P450 2E1 (CYP2E1), catalase is capable of oxidizing ethanol to acetaldehyde (Handler & Thurman, 1988a, 1988b). In previous studies, we demonstrated that the administration of fenofibrate to UChB (Universidad de Chile Bibulous) high alcohol-drinking rats produced a marked increase (2.5-fold) of catalase levels in the liver, together with a 10-fold increase (to 95 mM) in blood acetaldehyde levels when 1 g/kg ethanol was administered to these animals (Karahanian et al., 2014; Rivera-Meza et al., 2017). Consequently, a reduction of 60-70% in voluntary alcohol consumption was observed when it was measured in 24 h, but the reduction increased to 85-90% when consumption was measured in the first 2 h of the dark cycle (when animals drink proportionally more). Similarly, Blednov, Black, Benavidez, Stamatakis & Harris (2016) and Haile & Kosten (2017) reported that fenofibrate attenuated alcohol self-administration in mice and rats, respectively. Moreover, this effect was totally dependent on PPARα, since the administration of a PPARα antagonist or the use of knockout mice lacking this receptor, did not show any effect (Blednov et al., 2016)

Due to these encouraging previous results, in the studies presented here we studied whether the increase in catalase activity was solely responsible for the remarkable effect of fenofibrate in increasing blood acetaldehyde levels, or if there are also changes in ADH1 or ALDH2 protein levels and/or activities in the liver of these same animals. For this purpose, hepatic ADH1 and ALDH2 activities were measured, and protein levels were quantified by Western blot.

Methods

Animals

High-drinker UChB rats derived from the Wistar strain and bred selectively for their high alcohol intake were used in this study (Quintanilla, Israel, Sapag & Tampier, 2006). Two-month-old male rats were housed in individual cages in temperature- and humidity-controlled rooms under a regular 12-h light/12-h dark cycle. For 60 days, rats were offered the choice of a 10% (v/v) ethanol solution and water from 2 graduated tubes (or only water for controls). Food (Mardones rat formula, Alimentos Cisternas, Santiago, Chile) was provided ad libitum and the volume of water and ethanol solution consumed was recorded daily. After this time, ethanol consumption stabilized at ~7 g ethanol/kg/day. All procedures used in this study were revised by and in compliance with the Bioethics Committee on Animal Research, Faculty of Medicine, Universidad de Chile (Protocol CBA0767FMUCH).

Fenofibrate treatment

The treatment with fenofibrate has already been described in detail and the data reported here were obtained on the very livers (frozen at -80C) of animals used in such report (Rivera-Meza et al., 2017). Briefly, after 60 days of continuous (24 h/day) free choice between 10% (v/v) ethanol solution and water (or only water for controls), ethanol intake stabilized at ~7 g/kg/day. Then, animals were divided into 4 groups (n = 6 rats per group): one ethanol and one control (water) group were treated with micronized fenofibrate (Fibronil, Royal Pharma, Chile) administered orally as an aqueous suspension (10 mL/kg) at a dose of 100 mg/kg/day for 14 consecutive days. The other ethanol and control groups were treated with vehicle (water, 10 mL/kg, p.o.) for 14 consecutive days (Figure 1). Ethanol 10% (when pertinent), water and food were accessible *ad libitum* continuously 24 h/day. Ethanol and water intakes were daily recorded.

Quantification of ADH1 and ALDH2 levels in liver by Western blot and by enzymatic activity

After treatment with fenofibrate, ethanol was withdrawn for 24 hours and animals were sacrificed. Liver tissue was collected and homogenized in a pestle with 1% Triton X-100 in phosphate buffer [50 mM (pH 7.4)] containing a complete EDTA-free protease inhibitor cocktail (Pierce,

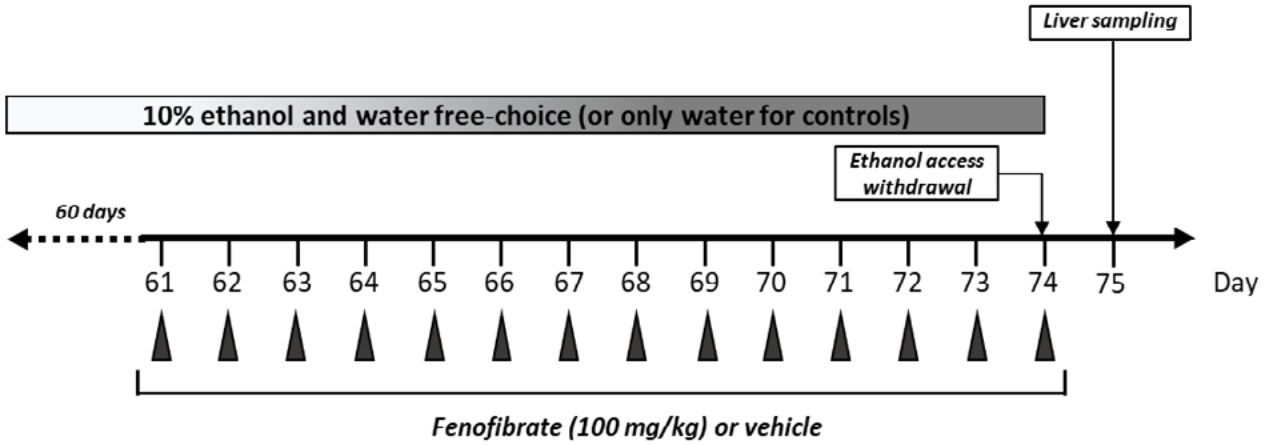

Figure 1. Graphical time schedule for the ethanol-drinking and fenofibrate treatment experiments. Twelve UChB male rats were given 24 h free choice between 10% v/v ethanol and water, and other twelve rats were given only water (as controls) for 60 days. Starting at day 61, each group was divided in two subgroups (n = 6) and 100 mg/kg/day of fenofibrate or vehicle respectively were given orally to each subgroup for 14 days. At the end of fenofibrate treatment, animals were deprived for ethanol access for 24 h, sacrificed and liver tissues were collected.

Rockford, IL, USA). Cell debris was removed by centrifugation and protein content was determined with the Micro BCA protein assay kit (Pierce, Rockford, IL, USA). Samples were analyzed by Western blot with ADH1 (Novus Biologicals NBP2-12550) and ALDH2 (Novus Biologicals NBP1-52051) primary antibodies. As loading control, b-actin levels were determined with Cell Signaling 8H10D10 primary antibody. The corresponding secondary antibodies were HRP-conjugated. Blotting membranes were revealed for chemiluminescence with Pierce ECL Western Blotting Substrate. The bands were quantified by densitometry with the ImageJ software.

Determination of alcohol dehydrogenase and aldehyde dehydrogenase activities in liver

The activities of ADH1 and ALDH2 were determined spectrophotometrically in the liver homogenates by the measurement of absorbance (340 nm) of reduced nicotinamide adenine dinucleotide (NADH) generated from NAD+ over time. ADH1 activity was measured as reported previously (Rivera-Meza et al., 2010): the assay was performed in 0.5 M Tris-HCl buffer (pH 8.0) containing 0.33 mM dithiothreitol, 24 mM semicarbazide, 5 mM NAD+, and 100 mg of total protein. The reaction was initiated by the addition of 10 mM ethanol. For ALDH2 activity, the reaction mixture contained 40 mM (pH 7.4) phosphate buffer, 4 mM dithiothreitol, 5 mM MgCl2, 10 mM 4-methyl-pyrazole, 0.8 mM NAD+, and 100 mg of total protein. The reaction was initiated with the addition of 14 mM propionaldehyde (Karahanian, Ocaranza & Israel, 2005). The specific activity of both enzymes was expressed as nmol of NADH/min/mg of protein, at 25 °C.

Satatistical analysis

All data were analyzed by one-way ANOVA and Newman-Keuls *post hoc* test.

Results

As shown in Fig. 2, fenofibrate (F) treatment produced a marked increase in the levels of ADH1 expression by Western Blot analysis, both in rats that consumed ethanol (E + F) and in controls that consumed water (W + F), in comparison with animals that were not treated with fenofibrate which received only water (W). The increase was much greater in the E + F group than in the W+F group (396% ± 18% *vs* 254% ± 19%, $p < 0.001$). In ethanol treated animals (E) that were not treated with fenofibrate (given water as vehicle), there was an increase in the expression of ADH1, compared to those who drank only water (W) (162% ± 9% *vs* 100% ± 10%, $p < 0.05$). These results highlight the effect of ethanol alone in increasing ADH1 levels in the liver. Conversely, as shown in Fig. 3, treatment with fenofibrate had no effect on ALDH2 protein levels, nor there was an effect of alcohol consumption on the levels of this enzyme.

The results of ADH1 and ALDH2 protein levels were in line with the measurement of enzymatic activities. As shown in Figure 4, treatment with fenofibrate produced increases in ADH1 activity in both rats that consumed alcohol and those that did not drink (425% and 475% respectively, compared to their controls). With respect to the activity of ALDH2, neither treatment with fenofibrate nor chronic consumption of alcohol produced differences in this enzyme (Fig. 5).

Discussion

The finding that treatment with fenofibrate results in an increase not only in the activity of catalase (Karahanian et al., 2014; Rivera-Meza et al., 2017) but also in liver ADH1 levels, is of value in understand the remarkable effects of this drug in producing an increase in blood acetaldehyde levels and finally a decrease in the voluntary consumption of alcohol in rats. The effect of the administration of fi-

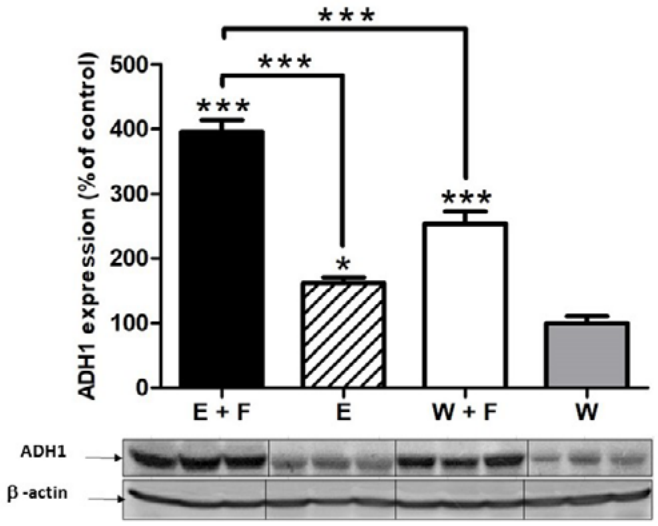

Figure 2. Effect of fenofibrate on the levels of liver alcohol dehydrogenase (ADH1). UChB rats that consumed ethanol (or water for controls) for 74 days were treated with fenofibrate 100 mg/kg/d during the last 14 days of consumption. The respective control groups were not given fenofibrate (but given water as vehicle). The levels of ADH1 were measured by Western blot and normalized with respect to the levels of b-actin. E + F: ethanol plus fenofibrate; E: ethanol; W + F: water plus fenofibrate; W: water only, n = 6 per group. The W group was set as 100%. The data were analyzed by one-way ANOVA and a Newman-Keuls *post hoc* test. * represents p ‹ 0.05 and *** represents p ‹ 0.001. The error bars correspond to SEM. Representative lanes of 3 samples of each group are shown below the graph.

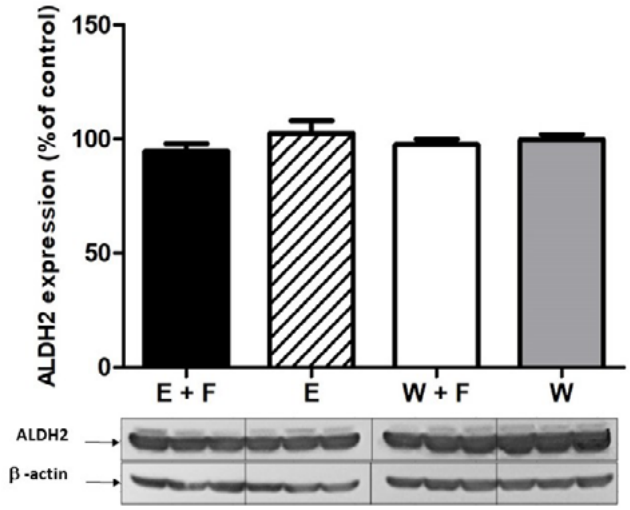

Figure 3. Effect of fenofibrate on the levels of liver aldehyde dehydrogenase (ALDH2). UChB rats that consumed ethanol (or water for controls) for 74 days were treated with fenofibrate 100 mg/kg/d during the last 14 days of consumption. The respective control groups were not given fenofibrate (but given water as vehicle). The levels of ALDH2 were measured by Western blot analyses and normalized with respect to the levels of b-actin. E + F: ethanol plus fenofibrate; E: ethanol; W + F: water plus fenofibrate; W: water only, n = 6 per group. The W group was set as 100%. The data were analyzed by one-way ANOVA and a Newman-Keuls *post hoc* test, no significant differences were found. The error bars correspond to SEM. Representative lanes of 3 samples of each group are shown below the graph.

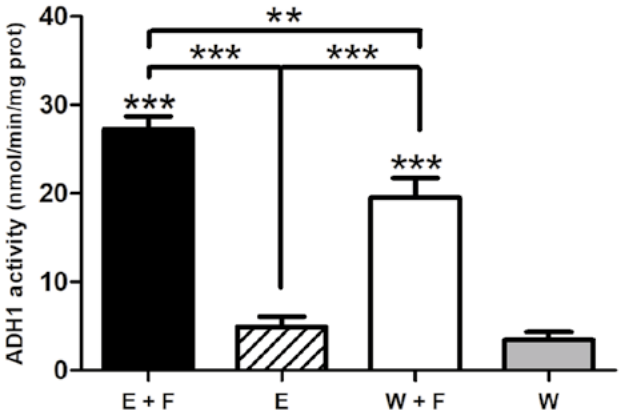

Figure 4. Effect of fenofibrate on the activity of liver alcohol dehydrogenase (ADH1). UChB rats that consumed ethanol (or water for controls) for 74 days were treated with fenofibrate 100 mg/kg/d during the last 14 days of consumption. The respective control groups were not given fenofibrate. ADH1 activity represents nmol of NADH/min/mg of protein, at 25 ºC. E + F: ethanol plus fenofibrate; E: ethanol; W + F: water plus fenofibrate; W: water only, n = 6 per group. The data were analyzed by one-way ANOVA and a Newman-Keuls *post hoc* test.

Note. ** represents p ‹ 0.01 and *** represents p ‹ 0.001.
The error bars correspond to SEM.

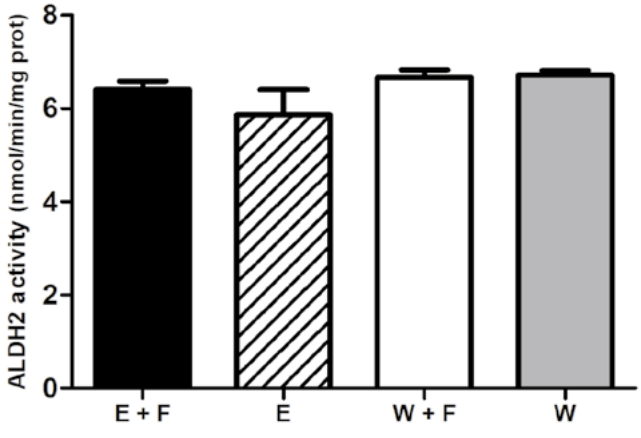

Figure 5. Effect of fenofibrate on the activity of liver aldehyde dehydrogenase (ALDH2). UChB rats that consumed ethanol (or water for controls) for 74 days were treated with fenofibrate 100 mg/kg/d during the last 14 days of consumption. The respective control groups were not given fenofibrate. ALDH2 activity represents nmol of NADH/min/mg of protein, at 25 ºC. E + F: ethanol plus fenofibrate; E: ethanol; W + F: water plus fenofibrate; W: water only, n = 6 per group. The data were analyzed by one-way ANOVA and a Newman-Keuls *post hoc* test. The error bars correspond to SEM. No statistically significant differences were found.

brates on the expression of ADH1 in the liver had been previously described by Kramer et al. (2003), who reported an increase in the transcription of this gene in rats treated with clofibrate. More recently, Ferguson, Most, Blednov & Harris (2014) reported increased ADH1 transcript levels in the liver after an 8-day treatment with fenofibrate or tesaglitazar (a dual PPARα and PPARγ agonist), but not bezafibrate (a pan PPAR agonist).

Even though the pioneering works of Lieber (1988) and others (Vidal, Perez, Morancho, Pinto & Richart, 1990)

show that hepatic ADH is not induced by alcohol consumption, there are also reports showing that intragastric administration of 13 g/kg/day of ethanol to rats produced increases in mRNA levels (Badger et al., 2000; Deaciuc, Arteel, Peng, Hill & McClain, 2004), protein and enzymatic activity (He, Ronis & Badger, 2002) of ADH1. In a similar way, we also found that chronic consumption of ethanol at ~7 g/kg/day *per se* produced an increase in hepatic ADH1 levels in rats. There may be an effect dependent on the dose of ethanol, since He et al. (2002) reported 3.3-fold increases in ADH1 levels with 13 g/kg/day of ethanol, while we detected a 1.6-fold increase in the presence of ≈ 7 g/kg/day. Interestingly, we observed that the simultaneous treatment with ethanol and fenofibrate produces the highest levels of ADH1, suggesting an additive effect between both treatments. One possible explanation for the increased expression of ADH1 by fenofibrate treatment might be the presence of a PPAR response element (PPRE) in the promoter of the rat Adh1 gene. We performed a search of the consensus PPRE sequence (Tzeng et al., 2015) in this promoter, but we did not find the presence of any PPRE. It is then possible that PPARα stimulates the expression of ADH1 by an indirect mechanism.

Unlike the reported in other studies where ALDH2 mRNA levels were decreased by clofibrate (Moffit et al., 2007) or tesaglitazar (Ferguson et al., 2014) in mice, we did not observe any change in ALDH2 protein levels produced by fenofibrate in our rat model of chronic alcohol consumption. This last result is interesting in terms of achieving an increase in the levels of acetaldehyde in blood. Crabb et al. (2001) reported a slight decrease in the expression of ALDH2 in the liver of clofibrate-treated rats and in mice treated with WY14643 (a PPARα agonist); however, WY14643 had exactly the same effect in PPARα-null mice, suggesting that such effect would not be mediated by PPARα.

The measurements of the enzymatic activities of ADH1 and ALDH2 in the liver showed similar results as the quantification of the proteins by Western blot: ADH1 showed the highest level of activity in the animals that drank alcohol and were treated with fenofibrate, with a lower increase in animals treated only with fenofibrate. Although in a previous work (Karahanian et al., 2014) we had reported that doses of fenofibrate 50 mg/kg/day did not alter the activity of ADH1 in the liver, in this work we perform the enzymatic measurements in rats treated with fenofibrate 100 mg/kg/day, suggesting that this higher dose of fenofibrate may be needed to generate increases in ADH1 activity. It has been demonstrated that a higher activity of ADH1 in the liver is directly related to a greater production of acetaldehyde when ethanol is consumed, both in animal models (Rivera-Meza et al., 2010; Rivera-Meza, Quintanilla & Tampier, 2012) and in humans (Lee et al., 2004). Genetic polymorphisms in humans have been identified to produce a forty-times more active ADH (ADH1B2) than the normal allele (Lee, Höög & Yin, 2004). Interestingly, this allele is relatively common among Asians, where carriers of this mutation have been shown to have a protective effect against alcoholism (Thomasson et al., 1991; Thomasson et al., 1994). Carrying a "faster" ADH would lead to an initial rapid accumulation of acetaldehyde (Quintanilla, Tampier, Sapag, Gerdtzen & Israel, 2007) when these individuals drink alcohol, causing the aversive effects described above. Data presented and discussed support the notion that the effect of fenofibrate in the increase of ADH1 activity would play an important role (in addition to catalase) in explaining the effectiveness of this drug in reducing alcohol consumption in rats.

Although it is true that the elevation of acetaldehyde levels in blood product of alcohol consumption account for several of the side effects of disulfiram, this drug *per se* presents other characteristics that explain its toxicity. Disulfiram is a highly unspecific drug that also inhibits the conversion of dopamine to noradrenaline, and the depletion of noradrenaline in the cardiovascular system potentiates acetaldehyde action on myocardial and vascular tissue to cause flushing, tachycardia and hypotension (Sinclair, Chambers, Shiles & Baldwin, 2016). In addition, disulfiram inhibits N-acetyl transferase and several members of the cytochrome family, thus increasing the toxicity of several other drugs (Frye & Branch, 2002). Another disadvantage of this drug is that there is marked intersubject variability in plasma levels of disulfiram and its metabolites (Faiman, Jensen & Lacoursiere, 1984) and because of its complex metabolic pathway there is a huge individual variability in the response (Mays et al., 1995).

As indicated above, disulfiram is ineffective in a significant percentage of patients to reduce ethanol intake (Christensen et al., 1991; Skinner et al., 2014; Yoshimura et al., 2014). We believe that there is an explanation for the lack of response to disulfiram in some individuals: although it is widely recognized that an increase in the peripheral levels of acetaldehyde generate ethanol-aversive effects, an idea that has been gaining strength is that acetaldehyde at the central level, when generated directly in the brain from ethanol, it has reinforcing properties towards alcohol consumption (Israel et al., 2013; Israel et al., 2015). Unlike the liver, ADH1 is not expressed in the brain; instead, catalase is the main enzyme responsible for oxidizing ethanol into acetaldehyde. Subsequently, as in the liver, ALDH2 is responsible for removing the accumulated acetaldehyde. In previous work, we have reported that by inhibiting the expression of catalase in the ventral tegmental area of the brain (involved in the release of dopamine in the limbic reward system) and therefore blocking the production of acetaldehyde in this region, the capacity of ethanol to generate addiction in UChB rats is inhibited (Karahanian et al., 2011). Similarly, increasing the removal of acetaldehyde by overexpression of ALDH2 gives the same effect (Karahani-

an et al., 2015). We believe that one of the disadvantages of disulfiram is that it can cross the blood-brain barrier (Hellström & Tottmar, 1982) and can inhibit ALDH2 activity in the brain. In this way, when the individual drinks alcohol catalase generates acetaldehyde in the brain that is not metabolized and, as we mentioned, could generate a reinforcing effect. In this way, we hypothesize that the aversion generated by peripheral acetaldehyde could be exceeded by the reinforcing effects at the central level. Therefore, to find a drug that is more effective than disulfiram in reducing alcohol consumption, this drug should ideally stimulate the production of acetaldehyde in the periphery and not in the brain. We think that fenofibrate would perfectly comply with these characteristics, since we have shown that it does not increase catalase activity in the brain.

Overall, these studies show that treatment with fenofibrate not only increases the activity of catalase in the liver of alcohol drinking rats as previously reported (Karahanian et al., 2014; Rivera-Meza et al., 2017), but also increases the ADH1 protein levels and its enzymatic activity, while ALDH2 remained unchanged. These results are valuable in understanding why fenofibrate has a remarkable effect of raising the levels of acetaldehyde in the blood when animals ingesting alcohol. However, more studies are needed to demonstrate the efficacy of fenofibrate on reducing alcohol consumption (for example, to study the possible effects at the central level that would decrease the motivation to drink, beyond the aversion generated by the peripheral acetaldehyde).

Acknowledgments

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Conflicts of interests

the authors have no conflicts of interest to declare.

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