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ORIGINAL ARTICLE

Modulation of ethanol effect on hepatocyte proliferation by polyamines

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Abstract An occurrence and a magnitude of alcoholic liver diseases depend on the balance between ethanol-induced injury and liver regeneration. Like ethanol, polyamines including putrescine, spermidine, and spermine modulate cell proliferation. Thus, the purpose of this study was to evaluate the relationship between effect of ethanol on hepatocyte (HC) proliferation and polyamine metabolism using the HepaRG cell model. Results showed that ethanol effect in proliferating HepaRG cells was associated with a decrease in intracellular polyamine levels and ornithine decarboxylase (ODC) activity. Ethanol also induced disorders in expression of genes coding for polyamine-metabolizing enzymes. The α-difluoromethyl ornithine, an irreversible inhibitor of ODC, amplified ethanol toxicity on cell viability, protein level, and DNA synthesis through accentuation of polyamine depletion in proliferating HepaRG cells. Conversely, putrescine reversed ethanol effect on cell proliferation parameters. In conclusion, this study suggested that ethanol effect on HC proliferation was closely related to polyamine metabolism and that manipulation of this metabolism by putrescine could protect against the anti-proliferative activity of ethanol.

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Introduction

Ethanol has been known to perturb function of the liver, main site of its metabolism. Thus, it increases risks of cirrhosis and/or hepatocellular carcinoma development through the imbalance between ethanol-induced injury and regenerative ability of remaining hepatocytes (HC). Recent data have indicated that ethanol inhibited the proliferation of osteoblasts (Vignesh et al. 2006), neuronal cells (Jacobs and Miller 2001), enterocytes (Koivisto and Salaspuro 1998), and HCs (Koteish et al. 2002; Norikura et al. 2007; Ramirez-Farias et al. 2008). However, in some conditions, ethanol has promoted the proliferation of HCs (Baumgardner et al. 2007) or gastric mucosal cells (Liu et al. 2008).

Cellular polyamines including putrescine, spermidine, and spermine are not exogenously absorbed via their transport system, but endogenously biosynthesized from ornithine due to ornithine decarboxylase (ODC). These aliphatic polycations play important roles in cell growth and differentiation through their interactions with polyanionic macromolecules such as DNA, RNA, and proteins (Gugliucci 2004; Wallace et al. 2003). Several studies in rat have indicated that hepatotoxicity of ethanol has been closely related to dysfunction in polyamine homeostasis. Indeed, acute and chronic ethanol exposure impaired putrescine uptake or increased putrescine conversion into spermidine/spermine and putrescine excretion into bile (Tanaka et al. 1993; Minuk and Gauthier 1993). This reduced putrescine levels and consequently retarded liver regeneration (Diehl et al. 1992). However, the liver

xenobiotic response differs between humans and animals. Thus, human hepatoma cell lines are now widely used for studies on liver metabolism and toxicity. We previously reported an inhibition of ethanol on HC proliferation in the model of HepaRG, which is so far a unique human bipotent cell line (Do et al. 2011). It could differentiate into HC and biliary epithelial cells (BC), paralleling liver development (Gripon et al. 2002). It is the sole cell line able to support hepatitis B virus infection similar to primary human HCs, express almost main liver enzymes (Gripon et al. 2005; Troadec et al. 2006; Aninat et al. 2006).

The above data encouraged us to undertake this study in HepaRG cells to determine a possible interrelation between effect of ethanol on HC proliferation and polyamine metabolism as well as a potential therapeutic application by manipulating polyamine metabolism in alcohol liver diseases. Precisely, we investigated the impact of ethanol on polyamine metabolism by measuring cellular polyamine levels, ODC activity, and expression of polyamine-metabolizing enzymes. Then, we evaluated effect of ethanol in the presence of α-difluoromethyl ornithine (DFMO), an irreversible inhibitor of ODC and exogenous putrescine.

Materials and methods

Materials

Ethanol and putrescine were purchased from Sigma-Aldrich Chimie (Saint Quentin Fallavier, France). DFMO was purchased from Merrel Down Research Institute (Strasbourg, France), William's E medium from Invitrogen (Paisley, UK), and fetal calf serum (FCS) from HyClone (Logan, UT). Other chemicals were obtained from Sigma-Aldrich Chimie (Saint Quentin Fallavier, France). All reagents were of first-grade purity and used without further purification.

Cell culture

HepaRG cells were cultured and seeded at 2.5×10^4 cm^{-2} in the culture microplates (Gripon et al. 2002). After 24-h incubation at 37 °C and 5 % CO_2 (day 1 of culture), the treatment with different reagents alone or combined was performed for 72 h and renewed every 24 h because of ethanol evaporation. The proliferating cells appeared with a homogenous epithelial phenotype.

Cell viability assay

Cell viability was evaluated as mitochondrial succinate dehydrogenase (SDH) activity, a marker of viable cells using MTT test (Denizot and Lang 1986). Briefly, SDH activity detected after 3-h incubation in culture medium without serum containing MTT (3-4,5-dimethyl-thiazol-2-yl-2,5 diphenyl-tetrazolium bromide) was converted into formazan dissolved in DMSO. The produced purple solution was spectrophotometrically measured at 535 nm by a Fusion™ Packard microplate reader (Packard Bioscience, Meriden, CT).

Protein assay

Total protein level of cell lysate was determined as previously described (Bradford 1976) by Protein Assay® (Biorad, Marnes-la-Coquette, France) using dilutions of bovine serum albumin as standard values.

Measurement of DNA synthesis

DNA synthesis was evaluated as tritium thymidine incorporation. 24 h before cell harvesting, [^{3}H] methyl-thymidine (Amersham, Uppsala, Sweden) was added to culture medium at a final concentration of 0.5 μCi/ml. The radioactivity of cell lysates was measured by a Packard Tricarb® 2100TR scintillation counter β (Perkin Elmer, Waltham, MA) and expressed as cpm/μg protein.

Cytotoxicity assay

Lactate dehydrogenase (LDH) activity of culture medium and cell lysate were measured by "Cytotoxicity detection LDH" kit (Roche, Mannheim, Germany). Extracellular LDH/total LDH ratio was considered an index of cytotoxicity.

Measurement of caspase 3/7 activity

Apoptosis was assessed through caspase 3/7 activity measured by "AMC caspase-3/7 assay" kit (AnaSpec, Fremont, CA). Cell lysates were incubated in the dark with substrate of caspases (Ac-DEVDAS-AMC) for 18 h at 37 °C with a slight agitation. Caspase 3/7 activity measured by fluorescence at 440/460 nm was expressed as RFU/g protein.

RNA extraction, cDNA synthesis, and real-time quantitative PCR

Total RNA extracted using "SV total RNA isolation system" kit (Promega, Madison, WI) was tested by spectrophotometrical dosage and electrophoretic separation on a 1.5 % agarose gel. cDNA was synthesized from 1 μg of total RNA by reverse-transcription using Moloney Murine Leukemia Virus reverse-transcriptase (Promega, Madison, WI). Gene expression was measured by real-time quantitative PCR performed

in triplicate on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, London, UK) using "qPCR Mastermix Plus for SYBR Green I" kit and human-specific primer (Eurogentec, Seraing, Belgium) designed with the assistance of Primer Express 1.0 software (Applied Biosystems, London, UK) from Genbank human mRNA sequences.

Determination of intracellular polyamines

Cells were collected in homogenization buffer (25 mM Tris HCl, pH 7.5, 0.1 mM EDTA, 1.0 mM dithiothreitol, and 0.02 % Brij35). Extracts were obtained by sonicating for 30 s in ice, then treating with concentrated perchloric acid for a final concentration at 0.2 N, homogenized, and placed at 4 °C overnight. After centrifugation at 13,000*g* for 10 min, the supernatants were collected for dansylation.

Dansylation procedure was performed according to Seiler using 1,10-diamino-dodecane (DAD) as internal standard (Seiler 1970). Aliquots (100 µl) of the perchloric supernatants reacted with four volumes of dansyl chloride in acetone (5 mg/ml) in the presence of solid sodium carbonate. Excessive dansyl chloride removed by a reaction with proline and acetone, and evaporation. Dansyl polyamines were extracted by 2 ml of cyclohexane, then evaporated to dryness. The residue was taken up in 200 µl acetonitrile.

LC/MSMS analyses of the polyamine content were performed on a Thermo Scientific TSQ Quantum-Ultra (ThermoFisher Scientific, Courtabeuf, France) coupled to a Triple Quadrupole Mass Spectrometer (Thermo Scientific, USA) equipped with an ESI interface supplied with Xcalibur software (Ducros et al. 2009).

Chromatography separation was achieved using a Hypersil Gold UPLC C18 column (ThermoFisher Scientific). The column temperature was set at 20 °C. The mobile phase consisted of 60 % solvent A (10 mM ammonium acetate in 0.1 % formic acid) and 40 % solvent B (acetonitrile in 0.1 % formic acid) at a flow rate of 530 µl/min. The injection volume was 10 µl. The mass spectrometer was operated in positive electrospray ionisation (ESI+) mode at 350 °C. The multiple reaction monitoring (MRM) analysis used the transitions 555.1/170.0, 845.0/170.3, 1135.0/1135.0, and 639.3/337.3 for putrescine, spermidine, spermine, and the internal standard DAD, respectively. The area under the ionic peak, determined for each selective ion, was corrected from the ionic intensity of the internal standard DAD. Polyamine concentrations were deduced from calibration curves and corrected from the protein content.

Measurement of ODC activity

The ODC activity was measured based on the putrescine formation as previously described (Gaboriau et al. 2005). Cells were collected in homogenization buffer for polyamine determination. After addition of reaction solution (50 mM Tris HCl, pH 7.5; 40 µM pyridoxal 5′-phosphate; and 1.25 mM dithiothreitol), cell lysates were incubated with ornithine for 1-h at 37 °C in a water bath under gentle agitation. The aliquots were recuperated before and after reaction, then precipitated with 0.2 M perchloric acid, and their putrescine level was measured in the perchloric supernatant as described above. ODC activity is expressed in µmol putrescine/g protein/h.

Statistical test

The results were presented as mean ± SD and analyzed by the statistical nonparametric Mann–Whitney test with the assistance of SPSS 15.0 for Windows (SPSS Inc., Chicago, IL). The threshold of significance was set at $P < 0.05$.

Results

Expression of genes encoding for polyamine metabolism enzymes

We determined the expression of polyamine metabolism genes in different steps of HepaRG differentiation process using human-specific primer designed by Primer Express 1.0 software (Applied Biosystems, London, UK), except spermidine/spermine N1-acetyltransferase 1 (SAT1) one (Linsalata et al. 2006) and presented in Table 1. The results showed that HepaRG cell line expressed almost cell enzymes of polyamine metabolism during the differentiation process (Table 2). The high values were obtained at day 6 or day 15 post-spreading. Freshly isolated human HCs (FIH) correspond to a pool of three different cell populations obtained from the biological resource center (CRB) of Rennes.

Ethanol-induced disorders in polyamine metabolism

We investigated the effect of 100 mM ethanol on polyamine content in proliferating HepaRG cells. Putrescine, spermidine, and spermine levels were lower in ethanol-treated cells compared to control (−43.0, −36.8, and −62.0 %, respectively, $P < 0.05$, Fig. 1a). Moreover, in the presence of 100 mM ethanol for 72 h, ODC activity was diminished (−40.8 % vs. control, $P < 0.05$, Fig. 1b).

We also examined the effect of ethanol on expression of polyamine-metabolizing enzymes. After 72-h treatment, ethanol (100 mM) insignificantly decreased the gene expression of ODC1, SMS, and SMOX (Fig. 1c). However, compared to control (100 %), ethanol exposure increased the gene expression of other enzymes involved in

Table 1 Specific primers of genes encoding for polyamines metabolism enzymes were designed by Primer Express 1.0 software from Genbank human mRNA sequences

Gene name	Symbol	Forward primer	Reverse primer
Ornithine decarboxylase antizyme 1	OAZ1	5′-CGAGCCGACCATGTCTTCAT-3′	5′-CCGGTCTCACAATCTCAAAG-3′
Ornithine decarboxylase	ODC1 (ODC)	5′-GTGCCACGCTCAGAAG-3′	5′-GGTACAGCCGCTTCCTACA-3′
Spermidine synthase	SRM (SPDS)	5′-CGTCTTCCGCAGTAAGACCTAT-3′	5′-TTGGCGATCATCTCCTGGTA-3′
S-adenosyl-methionine decarboxylase	AMD1 (SAMDC)	5′-GGTAATCAGTCAGCCAGATCAA-3′	5′-TTTGCAGTAACACCATCTTTCATG-3′
Spermine synthase	SMS (SPMS)	5′GAGGTGGAGACGGAGGCATA-3′	5′-TTCTTACACCCATCAATCACCAT-3′
Spermine oxidase	SMOX	5′-GATCCCCAGGACGTGGTT-3′	5′-TTCAGCATTGACTGGTTTATCGT-3′
Spermidine/spermine N1-acetyltransferase 1	SAT1 (SSAT)	5′-GGTCCGCAAAGGGAAGAAA-3′	5′-TGCCAATCCACGGGTCATA-3′
Polyamine oxydases	PAOX (PAO)	5′-GTAGGCTGGGACCGTCATT-3′	5′-AAGGTGGCCCTGGTTACCA-3′
Specific human 18Sl	18S	5′-CGCTCTACCTTACCTACCTGG-3′	5′-ATGGCTTAATCTTTGAGACAAG-3′

Table 2 Expression of polyamine metabolism enzymes in different steps of HepaRG differentiation process

	OAZ1	ODC1	SRM	AMD1	SMS	SMOX	SAT1	PAOX
FIH	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
HepaRG cells (days)								
Day 3	65.5	407.0	293.7	149.2	114.2	103.1	15.7	2.1
Day 6	206.6	389.6	278.8	124.0	172.8	174.9	40.3	10.1
Day 15	58.2	386.6	932.9	541.1	69.2	112.1	24.2	29.9
Day 30 (DMSO)	55.1	520.2	97.8	97.6	46.7	79.8	63.1	5.6

Results were presented as percentages compared to freshly isolated human hepatocytes (FIH) arbitrarily set at 100 % of one representative experiment. FIH correspond to a pool of three different cell populations obtained from the biological resource center (CRB) of Rennes

polyamine metabolism such as OAZ1 (+51.7 %), SRM (+51.2 %), AMD1 (+136.3 %), SAT1 (+103.5 %), and PAOX (+44.4 %) ($P < 0.05$, Fig. 1c).

Amplification of the ethanol anti-proliferative effect by polyamine depletion with α-difluoromethyl ornithine (DFMO)

To determine whether disorders in ODC activity could be one of underlying mechanisms of ethanol-induced toxicity, we measured its effect in the presence of DFMO, an inhibitor of ODC activity. After 72 h, compared to control, DFMO (5 mM) itself inhibited the proliferation of HepaRG cells through its reductions in cell viability (−27.1 %) and DNA synthesis (−15.8 %), associated with a cytotoxicity leading to an increase in LDH release (+27.9 % vs. control, $P < 0.05$, Fig. 2a). When DFMO was added into culture medium containing 100 mM ethanol, it amplified ethanol-induced toxicity in proliferating HepaRG cells (Fig. 2a): cell viability (−40.1 vs. −18/6 % of ethanol alone, $P < 0.05$), total protein levels (−57.4 vs. −28.2 %, $P < 0.05$) and DNA synthesis (−29.5 vs. 19.5 %, $P < 0.05$). However, the differences in LDH release and caspase 3/7 activity were not significant ($P > 0.05$, Fig. 2a). This amplification was associated with changes in polyamine metabolism. Indeed, compared to treatment with ethanol alone, that of ethanol and DFMO had a simultaneous reduction in spermidine and spermine levels (−15.4 and −8.1 %, respectively, $P < 0.05$, Fig. 2b). A decrease in ODC activity in the combination of ethanol with DFMO (−36.7 % vs. ethanol alone, $P < 0.05$, Fig. 2b) was not a significant change in putrescine level ($P > 0.05$, Fig. 2b). The presence of DFMO also modified the effect of ethanol on gene expression of enzymes involved in polyamine conversion: enhancement of SRM, AMD1, SMS, and PAOX activity (+11.9, +8.1, +16.5, and +62.7 %, respectively, $P < 0.05$, Fig. 2c); impairment of SMOX and SAT1 expressions (−22.7 and −57.5 %,

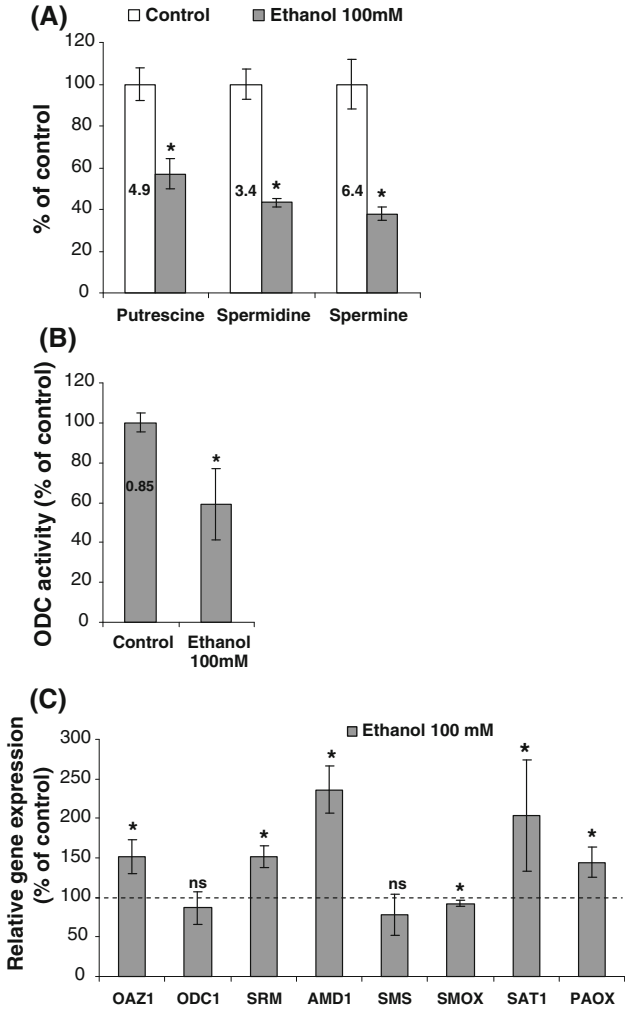

Fig. 1 Effect of ethanol on polyamines metabolism in the proliferating HepaRG cells. Cellular polyamines content (**a**) was measured by LC/MSMS system after dansylated using 1,10-diamino-dodecane as internal standard. Ornithine decarboxylase (ODC) activity (**b**) was analyzed based on the putrescine formation as described in "Materials and methods" section. The values on control bars represent polyamines levels (µmol/g protein) and ODC activities (µmol putrescine/g protein/h) in control HepaRG cells. Expression of polyamine-metabolizing enzymes (**c**) was assessed by real-time quantitative PCR, calculated after normalization using 18S and expressed as percentages compared to control culture (*dashed lines* at 100 %). Characters * and ^{ns} on histograms represent $P < 0.05$ and $P > 0.05$, respectively, according to Mann–Whitney test for the difference between ethanol-treated culture and control one

respectively, $P < 0.05$, Fig. 2c). The mRNA levels of OAZ1 and ODC were slightly decreased (Fig. 2c).

Reversion of the ethanol anti-proliferative effect by exogenous putrescine

As shown in Fig. 3a, after 72 h, 50 µM putrescine reversed partially the effect of 100 mM ethanol on cell viability (+19.0 %), protein content (+18.0 %), and DNA synthesis (+20.0 %) ($P < 0.05$). However, putrescine did not change ethanol effect on LDH leakage but increased caspase 3/7 activity (+30.5 %, $P < 0.05$, Fig. 3a). This was associated with a reversion of ethanol effect on cellular putrescine and spermidine levels (+49.9 and +36.4 %, respectively, $P < 0.05$, Fig. 3b) as well as ODC activity (+5.5 %, $P < 0.05$, Fig. 3b). The results also indicated that the presence of 50 µM putrescine reversed ethanol-induced rises in SMS and PAOX expressions (−50.5 and −35.0 %, respectively, $P < 0.05$, Fig. 3c). However, an amplification of ethanol effect on the mRNA levels of SMOX and SAT1 was observed (−32.5 and +251.8 %, respectively, $P < 0.05$, Fig. 3c).

Discussion

Polyamine metabolism during the differentiation of HepaRG cells

Our results show for the first time that the human hepatoma HepaRG cells express almost genes involved in polyamine metabolism during their differentiation. The high values obtained for ODC1, SRM, and AMD mRNA levels, at day 6 or day 15 post-spreading, were considerably increased compared to FIH and corresponded to a high-polyamine need for cell division, especially for hyperproliferation of tumor cells (Gugliucci 2004; Wallace et al. 2003). This was well correlated with our observation in which polyamine level and ODC activity in HepaRG cells gradually decreased from proliferation to differentiation state (data not shown). This also corresponded to several studies showing a high-ODC activity in the proliferation state compared to the differentiation one (Chen et al. 1982; Ewton et al. 1984; Verma and Sunkara 1982). Therefore, the HepaRG cell line could be used a valuable human-relevant in vitro model for studies on liver polyamine metabolism.

Ethanol effect on polyamine metabolism in HepaRG cells

Ethanol diminished intracellular polyamine content and ODC activity in proliferating cells. These results were similar to previous works reporting that ethanol decreased polyamine content in different models (Tanaka et al. 1993; Norikura et al. 2007). This could be from ethanol-induced decrease in ODC activity and enhancement of putrescine degradation via DAO pathway as well as polyamine export via their acetyl derivatives (Gerner et al. 1986). This speculation totally corresponded to an over-expression of SAT1 and PAOX in our study. The reduction in ODC activity could be explained by an increase of

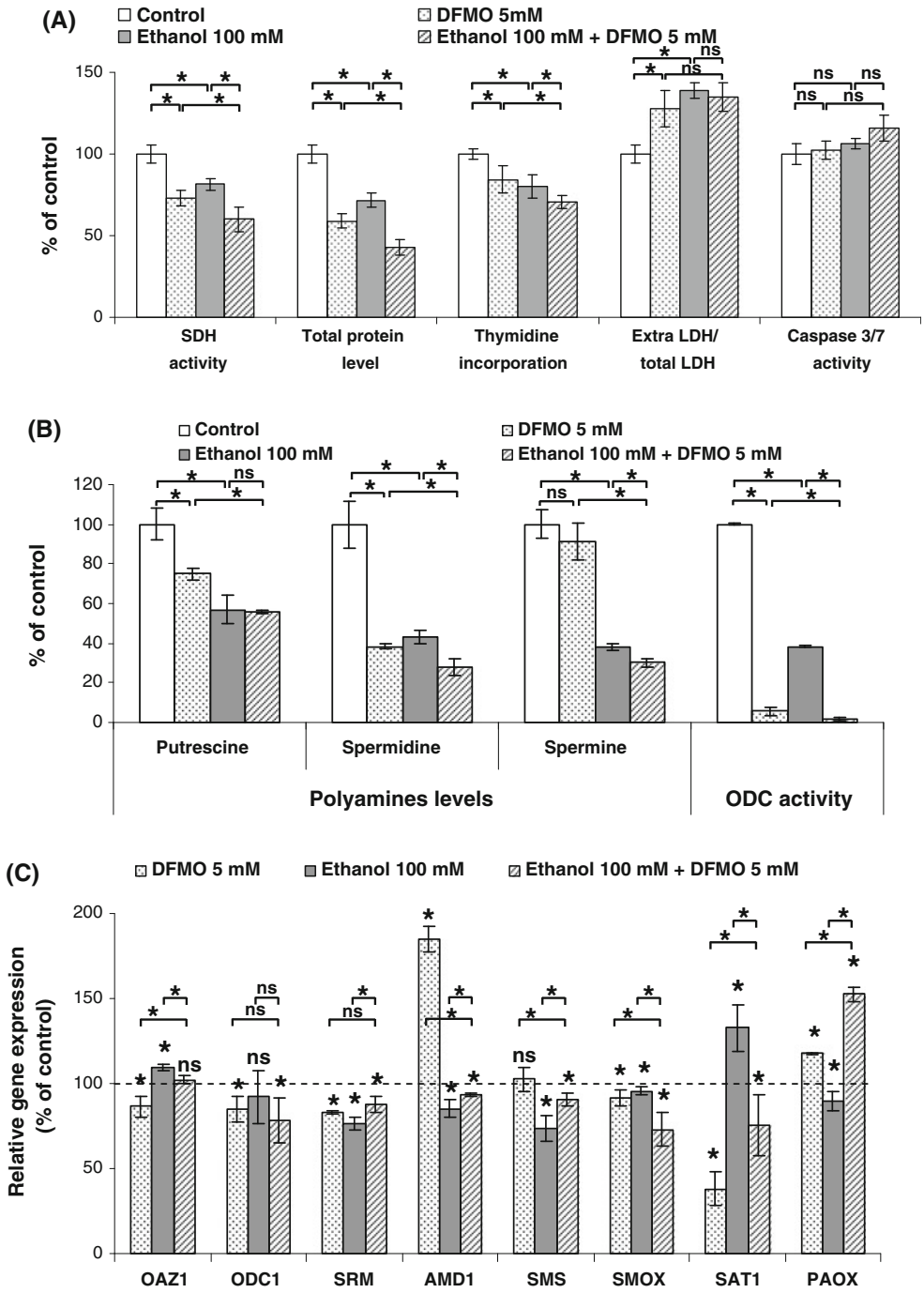

Fig. 2 Amplification of ethanol effect in the proliferating HepaRG cells by α-difluoromethylornithine (DFMO). This effect expresses at the levels of cell viability, total protein, DNA synthesis, LDH release, caspase 3/7 activity (**a**); polyamines content and ornithine decarboxylase activity (**b**) as well as expression of genes involved in polyamine-metabolizing enzymes (**c**) measured by assays as described in "Materials and methods" section. Relative gene expression is expressed as percentages compared to control culture (*dashed lines* at 100 %). Characters * and ^{ns} on histograms represent $P < 0.05$ and $P > 0.05$, respectively, according to Mann–Whitney test for the difference between two culture conditions (control/DFMO 5 mM vs. ethanol ± DFMO 5 mM)

OAZ1, which associated with ODC to form an OAZ–ODC complex degraded by 26S proteosome (Yatin et al. 1999). The impairments in polyamine levels have stimulated the other pathway of their synthesis via AMD1, SRM, and SMS since an overexpression of AMD1 and SRM was observed in this study. Although ODC mRNA expression did not significantly change, its activity remarkably decreased because ethanol reduced the level of pyridoxal 5-phosphate—cofactor of ODC (Sessa and Perin 1997).

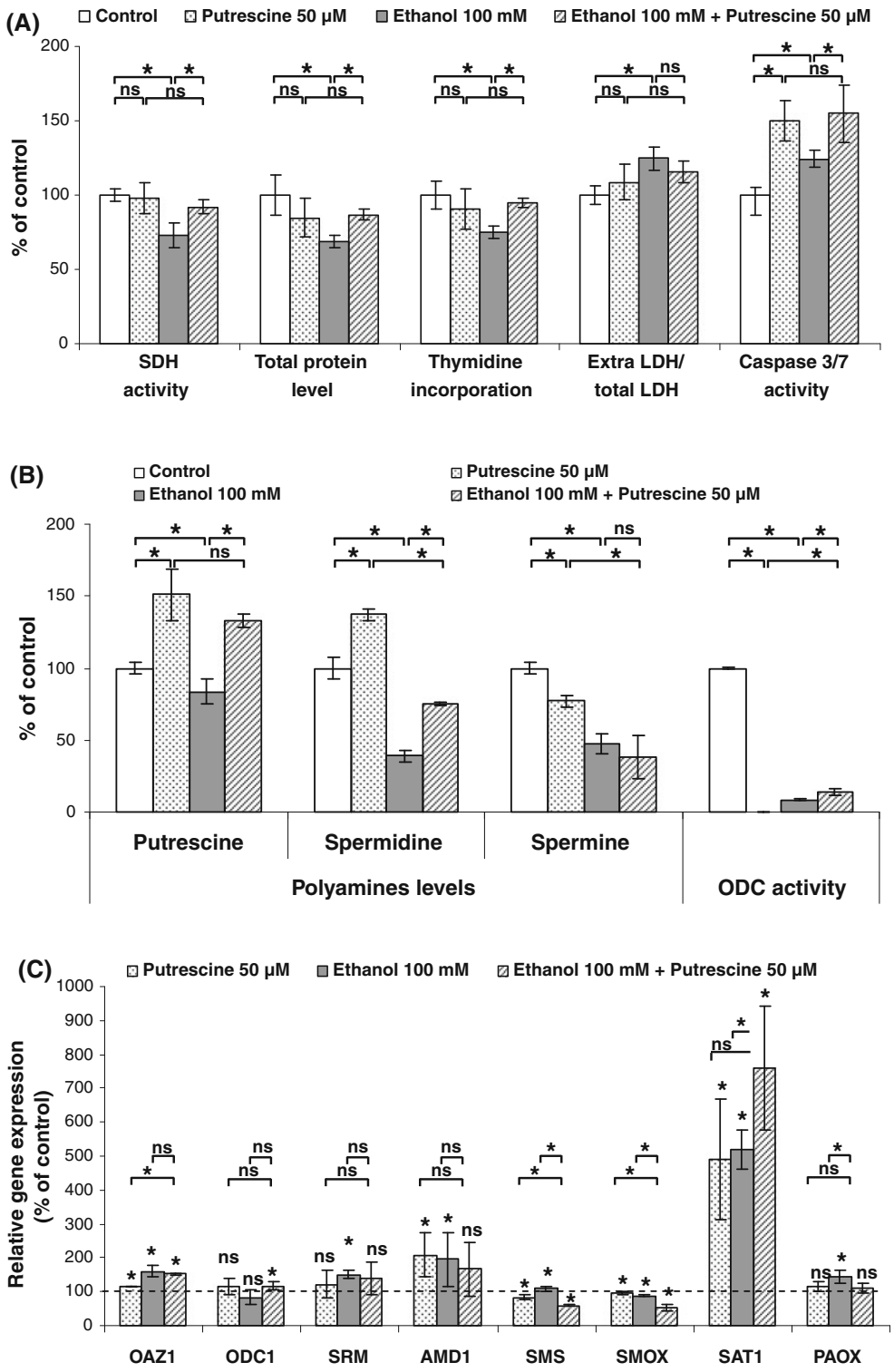

Fig. 3 Reversion of ethanol effect in proliferating HepaRG cells by putrescine. Attenuation of 100 mM ethanol toxicity at the level of cell viability, protein level, DNA synthesis, associated with a rise in caspase 3/7 activity without alteration in LDH release by 50 µM putrescine (**a**) due to a reversion of reductions in polyamines levels and ornithine decarboxylase activity (**b**) as well as some changes in expression of polyamine-metabolizing enzymes (**c**). Parameters were measured by assays as described in "Materials and methods" section. Relative gene expression is expressed as percentages compared to control culture (*dashed lines* at 100 %). Characters * and ^{ns} on histograms represent $P < 0.05$ and $P > 0.05$, respectively, according to Mann–Whitney test for the difference between two culture conditions (control/DFMO 5 mM vs. ethanol ± DFMO 5 mM)

Modulation of ethanol anti-proliferative effect by polyamine metabolism manipulation

The polyamine depletion amplified ethanol reduction in ODC activity, spermidine, and spermine levels without change in putrescine content. The ODC activity loss in cells exposed to ethanol plus DFMO could be responsible for the decrease in polyamine levels and increased the anti-proliferative effect, similar to Klein's study (Klein and Carlos 1995). Ethanol leads to a too low-putrescine level, thus DFMO addition did not change it, but enhanced the reductions in spermidine and spermine.

The ethanol-induced reductions in polyamine levels associated with its anti-proliferative effect raised the question of a possible reversion of ethanol toxicity by exogenous polyamines. In this study, putrescine addition reversed the inhibitory effect of ethanol on HepaRG cell proliferation due to its reversion of the reductions in putrescine and spermidine levels as well as ODC activity. This confirmed a protection of putrescine against ethanol toxicity previously reported in rat HCs (Diehl et al. 1990; Norikura et al. 2007) and suggested a clinical therapeutic application of putrescine in alcohol liver diseases. There was an insignificant difference between ethanol alone and combined with putrescine in LDH release and caspase 3/7 activity. This could be explained by that putrescine catabolism produced aldehydes and hydrogen peroxide, leading to cytotoxicity and apoptosis (Seiler and Raul 2005). Similarly, the gene expression of polyamine-metabolizing enzymes was not changed, except SMS and SMOX. Thus, post-translational regulation of ODC by putrescine contributed to the reversion of ethanol effect on ODC activity and polyamine levels; the result was an attenuation of ethanol hepatotoxicity.

In conclusion, this study showed that the HepaRG cell line could be used as a model for studies on polyamine metabolism because it expressed almost all cell polyamine-metabolizing enzymes during the differentiation process. The results demonstrated that effect of ethanol on HC proliferation was associated with a modulation in polyamine metabolism measured as a decrease in polyamine levels, ODC activity and a rise in mRNA levels of several polyamine-metabolizing enzymes. The anti-proliferative effect of ethanol was amplified in situation of polyamine depletion by DFMO and reversed by putrescine. Our data suggested that disorders of polyamine metabolism contributed to effect of ethanol and that putrescine could protect against ethanol-induced hepatotoxicity.

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Conflict of interest All authors do not have conflict of interest.

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