Elevation of EPO production is important for erythropoiesis as well as cell viability and overall health. The most effective factor for increasing EPO production is hypoxia. However, hypoxia is toxic for various organs and induces neuronal apoptosis. Therefore, it is thought that using hypoxia to increase EPO production in therapeutic settings is a flawed concept. However, inducing EPO production under normoxic conditions is difficult. There are reports that cobalt and quercetin are capable of increasing HIF levels and thereby increasing EPO production. Monitoring increases in HIF levels are frequently targeted in the development of drugs that promote EPO production under normoxic conditions; however, this is not a practical approach for this objective.

Hydroxylation of HIF-α by PHDs renders HIF-α unstable, leading to degradation by the von Hippel-Lindau (VHL) protein. HIF levels are frequently targeted in the development of drugs that promote EPO production under normoxic conditions; however, this is not a practical approach for this objective.
normoxia, we examined the influence of ethanol on EPO production in HepG2 cells, which have an EPO-producing ability.

Materials and methods

Materials

HepG2 cells were provided by RIKEN BRC through the National Bio-Resource Project of MEXT, Japan. Primers for real-time reverse transcription-polymerase chain reaction (RT-PCR) were purchased from Genedesign Co. (Osaka, Japan). Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Nissui Pharmaceuticals Co. (Tokyo, Japan). Glucose and pyruvate free DMEM was purchased from Sigma-Aldrich (St. Louis, MO) and 2.5 mM glucose with or without 1.25 mM sodium pyruvate was added. The SIRT1 inhibitor EX-527 was purchased from Sigma-Aldrich. Standard laboratory chemicals and reagents were purchased from Wako Pure Chemical Co. (Osaka, Japan). To amplify human mRNA, we designed the following pairs of primers: 5'- AGTGAGTGGCAGTGATGGCA-3' and 5'- GACGAGGTGAGGCTTGTTAGT-3' for EPO mRNA, spanning positions 845 to 911 [30]. To amplify rat mRNA, we designed the following pairs of primers: 5'- GGTAGTGCTGTTGGAATACCTGACT -3' and 5'- GTCTAGCAAGGGCCAGCATAA -3' for SIRT1 mRNA, spanning positions 483 to 545 (accession number, XM_003751934).

Animals

Male Wistar rats (7-week-old) were purchased from Nippon SLC Co. (Shizuoka, Japan). The experimental design and methods for animal care were pre-approved according to the guideline for animal experimentation of the animal care committee of Osaka Prefecture University. Liver and kidney cells of 9-week-old rats were harvested by tissue perfusion with 0.05% collagenase in Ringer’s solution. Liver and kidney cells were seeded at a concentration of 2.0 × 10⁵ cells/ml in DMEM containing 10% fetal bovine serum.

Cell culture

HepG2 cells were sub-cultured in DMEM containing 10% fetal bovine serum in 95% air and 5% CO₂ at 37°C. When HepG2 cells reached a concentration of 106 cells/ml, they were used for culture experiments. Cells used for experimentation had >95% viability as determined by trypan blue-exclusion assay. In culture experiments, 1 ml of HepG2, liver, and kidney cells (final concentration of 2.0 × 10⁵ cells/ml) were sub-cultured for 24 h in 5% CO₂ at 37°C. Sub-cultured cells were incubated in the presence of various concentrations of ethanol and other chemicals and cultured for an additional 6 h. Hypoxic conditions were produced by culturing in 5% O₂, 90% N₂, and 5% CO₂ at 37°C for 6 h. Cells were collected for measurement of mRNA concentrations by RT-PCR and protein levels of HIF-1α, HIF-2α and SIRT1. All samples were tested in duplicate.

Determination of mRNA levels

RNA was collected from whole cultured cells using a Gen Elute Mammalian total RNA kit (Sigma-Aldrich). The relative levels of specific mRNAs were determined by RT-PCR using a PowerSYBR Green RNA-to-Ct 1-Step Kit (Applied Biosystems, Foster City, CA) on an ABI StepOnePlus Real-Time PCR System (Applied Biosystems, Carlsbad, CA). The following PCR program was used: 48°C for 30 min, 95°C for 10 min; 40 cycles of 95°C for 15 sec and 60°C for 60 sec. After PCR, dissociation curves were constructed to confirm the amplification of uniform products. Quantification of mRNA was performed using the comparative delta CT method. GAPDH mRNA was used as the control, and the ratio of each experimental mRNA to GAPDH was calculated. The values of mRNAs are provided as values relative to the value of untreated cells. Each mRNA sample was measured twice and the levels of EPO, HIF-1α and GAPDH were measured from the same samples.

Determination of metabolites and erythropoietin

Lactate and pyruvate contents in HepG2 cell were measured spectrophotometrically with an enzymatic assay [33]. HepG2 cells were washed twice with ice-cold phosphate buffered saline, and washed cells terminated by the addition of 1 ml of 5% (v/v) HClO₄. Each sample was neutralized by K₂CO₃ and collected supernatant by centrifuging for assay. Ethanol concentrations in the culture media were measured fluorescence spectrophotometrically using an enzymatic assay [34]. Concentrations of erythropoietin in culture media were determined using an ELISA kit (ABnova, Taipei, Taiwan).

Western blot analysis

For the identification of HIF-1α and 2α protein levels, proteins were extracted from whole cell lysates in RIPA buffer and were separated by 10% SDS-PAGE before being transferred onto polyvinyl difluoride membranes (Biorad, Hercules, CA). The following PCR program was used: 48°C for 30 min, 95°C for 10 min; 40 cycles of 95°C for 15 sec and 60°C for 60 sec. After PCR, dissociation curves were constructed to confirm the amplification of uniform products. Quantification of mRNA was performed using the comparative delta CT method. GAPDH mRNA was used as the control, and the ratio of each experimental mRNA to GAPDH was calculated. The values of mRNAs are provided as values relative to the value of untreated cells. Each mRNA sample was measured twice and the levels of EPO, HIF-1α and GAPDH were measured from the same samples.

Statistical analysis

Statistical significance was determined using the Tukey-Kramer method.

Results

Figure 1 shows the effects of ethanol on EPO mRNA expression in normoxic HepG2 cells. Ethanol at 300 μM increased EPO mRNA expression; however, the EPO levels decreased at ethanol concentrations greater than 300 μM and were not significantly elevated at concentrations greater than 1000 μM.
Figure 1: Effect of ethanol on erythropoietin mRNA expression in HepG2 cells. HepG2 cells were cultured in 5% CO₂ at 37°C for 6 h in the absence or presence of ethanol and EPO mRNA levels were normalized to expression in the absence of ethanol, with GAPDH mRNA used as the control. Hypoxic condition involved culturing in 5% O₂, 90% N₂, and 5% CO₂ at 37°C for 6 h. Each mRNA sample was measured twice. Values represent the mean ± S.D. (n = 4). Asterisks indicate significant differences as compared with the values for 0 µM of ethanol (p<0.05).

Hypoxia increased EPO mRNA levels three-fold relative to normoxia. Ethanol treatment, at all concentrations, did not affect cell viability as determined by trypan blue staining (data not shown). Ethanol concentration in the culture media decreased in a time-dependent manner, and did not significantly decrease up to 6 h after the addition of 2000 µM ethanol (Table 1).

Table 1: Changes of ethanol concentration in culture media. Values represent the mean ± S.D. (n = 4). Asterisks indicate significant differences as compared with the values for 0 hour of ethanol addition (p<0.05).

| Time after addition (hour) | Ethanol addition |
|---------------------------|------------------|
|                           | 300 µM           | 2000 µM         |
| 0                         | 309.0 ± 13.7     | 1990 ± 58       |
| 2                         | 265.9 ± 19.8     | 1920 ± 69       |
| 4                         | 202.5 ± 15.3*    | 1890 ± 78       |
| 6                         | 162.2 ± 11.8*    | 1850 ± 75       |
| 8                         | 115.9 ± 10.5*    | 1820 ± 81*      |
| 10                        | 75.0 ± 8.9*      | 1790 ± 84*      |
| 12                        | 35.7 ± 5.9*      | 1750 ± 94*      |

Ethanol metabolism by alcohol dehydrogenase produces reducing equivalents and affects the cellular redox state. The ratio of lactate to pyruvate is shown in Figure 2A as an index of the cytosolic redox state. The ratio of lactate to pyruvate significantly increased in an ethanol dose-dependent fashion. Hypoxia also increased the ratio of lactate to pyruvate to the same degree as 300 µM ethanol (Figure 2A).

Since the effect of ethanol on EPO mRNA expression was not observed at high ethanol concentrations, the effects of 2000 µM ethanol were compared to 300 µM ethanol, which showed increased EPO mRNA expression. The lactate concentration was increased at 1 h after addition of 300 and 2000 µM ethanol and remained stable for up to 6 h after addition (Figure 2B). The pyruvate concentration was increased temporally by 30 µM ethanol and decreased by 2000 µM ethanol (Figure 2C).

To clarify whether the effect of 300 µM ethanol on promoting EPO mRNA expression is due to the influence of the ethanol metabolite acetate, the effect of equivalent concentrations of acetate was examined. Acetate alone did not alter either EPO mRNA expression (Figure 3A) or the ratio of lactate to pyruvate (Figure 3B). In the presence of pyruvate-free DMEM, increases in the ratio of lactate to pyruvate by 300 µM ethanol were elevated in comparison with normal DMEM, while ethanol-induced EPO mRNA expression was inhibited (Figure 3). Methanol, which like ethanol produces NADH, increased EPO mRNA expression at a concentration of 300 µM; however, no change was observed with 2000 µM methanol.
Figure 3: Effect of acetate and the absence of pyruvate on EPO mRNA expression and the lactate to pyruvate ratio in HepG2 cells. HepG2 cells were cultured in 5% CO2 at 37°C for 6 h in the absence and presence of 300 µM ethanol, 300 µM acetate, in the absence of pyruvate (+ ethanol), or 300 µM and 2000 µM methanol. After 6 h, cells were harvested and EPO mRNA expression and whole cell lactate and pyruvate contents were determined. Each sample was measured twice. Values represent the mean ± S.D. (n = 4). Asterisks indicate significant differences as compared with the values for none (0 µM of ethanol) (p<0.05). Crosses indicate significant differences as compared with the values for 300 µM of ethanol (p<0.05).

Figure 4 shows the effects of 300 µM and 2000 µM ethanol on HIF-1α and 2α protein contents in HepG2 cells. Levels of both HIF-1α and HIF-2α mRNA were increased in the presence of 300 µM ethanol, while no changes were observed in the presence of 3000 µM ethanol. Similarly, cellular HIF-1α and HIF-2α contents were also increased by 300 µM ethanol only. Total PHD content was not changed by treatment with either 300 or 2000 µM ethanol (data not shown).

Figure 5: Effect of ethanol on sirtuin-1 mRNA expression and protein content in HepG2 cells.

SIRT-1 is a transcriptional factor related to both HIF and cytosolic redox state. Because redox dependent SIRT1 activity influences the quantity of HIF [35,36] the effect of ethanol on SIRT1 was examined. Figure 5 shows the effects of 300 µM and 2000 µM ethanol on SIRT1 mRNA expression and cellular SIRT1 content. At 300 µM, ethanol increased SIRT1 mRNA expression, while 2000 µM ethanol had no effect. Cellular SIRT1 content was also increased at 300 µM ethanol only.
HepG2 cells were cultured as in Figure 4 and sirtuin-1 mRNA expression and protein content were measured. Each sample was measured twice. Values represent the mean ± S.D. (n = 4). Asterisks indicate significant differences as compared with the values for 0 µM of ethanol (p<0.05).

To confirm that SIRT1 regulates EPO production, the effect of the SIRT1 inhibitor EX-527 on EPO production was measured (Figure 6). EX-527 treatment inhibited the elevation in EPO mRNA expression produced by 300 µM ethanol. EX-527 also inhibited the increases in HIF-1α and 2α by 300 µM ethanol (data not shown). However, EX-527 did not affect the ratio of lactate to pyruvate (Figure 6A).

To examine the influence of ethanol on EPO production in the kidneys and liver, which are the EPO-producing organs in adults, the effect of ethanol on liver and kidney primary cells was determined. Figure 7 shows the effect of 300 µM ethanol on rat primary hepatocytes and kidney cells. In both cell types, 300 µM ethanol increased SIRT1 and EPO mRNA expression and EPO concentration in media.

Liver and kidney cells were cultured in 5% CO2 at 37°C with 100 µM ethanol for 6h to determine sirtuin-1 (A) and EPO (B) mRNA expression or for 24 hrs to determine EPO production (C). Each sample was measured twice. Values represent the mean ± S.D. (n = 4).
Asterisks indicate significant differences as compared with the values for 0 µM of ethanol (p<0.05).

Discussion

We showed that low concentrations of ethanol increased EPO production in HepG2 cells and rat primary hepatocytes or kidney cells. It is generally known that acetaldehyde produced by ethanol metabolism is hepatotoxic. Many studies on ethanol-induced liver toxicity have been performed, however, most cytotoxicity experiments have used ethanol more than 10 mM [26,27,37,38]. Acetaldehyde produced at low ethanol concentrations (300 µM) is immediately metabolized to acetic acid [26-28], and it is thought that the majority of the toxicity is avoided. Acetic acid is a substrate for various metabolic pathways, including the TCA cycle. Because increased EPO mRNA expression in response to acetic acid was not observed, it was hypothesized that ethanol-induced reducing equivalent, produced by ethanol metabolism, was involved in promoting EPO production.

Highly concentrated ethanol greatly increased the ratio of lactate to pyruvate and committed the cells to a reducing environment, thereby eliminating the effect of ethanol on increasing EPO production. In the absence of pyruvate, 300 µM ethanol increased the ratio of lactate to pyruvate to a greater extent than hypoxia, whereas EPO mRNA expression did not increase. These results suggest that a change in cytosolic redox state to the same extent as hypoxia was effective in promoting EPO production.

Gambini et al. [24] reported that an ethanol addition promoted SIRT1 mRNA expression. However, because they used 10 mM ethanol, it is unclear whether the mechanism is the same as in our experiment.

Because the effect of 300 µM ethanol on EPO production was greatly inhibited by the SIRT1 inhibitor EX527, it was thought that the promotion of EPO production with 300 µM ethanol was dependent on SIRT1. In fact, addition of 2000 µM ethanol decreased NAD+ content and did not alter the levels of SIRT1 mRNA and SIRT1 protein, resulting in no change in EPO production. It is reported that SIRT1 interacts with HIF [35,36]. SIRT1 inhibits the inactivation of HIF-α by PHD via deacetylation of HIF-α and enhances the effects of HIF [35,36]. In this study, both HIF-1α and 2α levels were increased by 300 µM ethanol. Therefore, it was thought that increased SIRT1 activity, induced by the addition of low ethanol concentrations, promotes EPO production by activation of HIF. In this study, the effects of ethanol on HIF-1α and 2α expression were similar, and differences in the role of HIF1 and 2 in ethanol-induced EPO production are not clear. On the other hand, it has been reported that HIF influenced SIRT1 [39]. Because EX-527 inhibited HIF induction and EPO production induced by 300 µM ethanol, it was thought that regulation of SIRT1 by HIF was minimal. It has also been reported that reducing conditions in cells induces HIF [22,24]. These reports are consistent with the results of the present study.

Promotion of EPO production by low ethanol concentrations was observed with HepG2 cells as well as cultured primary liver and kidney cells. Therefore, it is expected that low ethanol concentrations will promote in vivo EPO production. It is well known that chronic liver damage is a consequence of ethanol toxicity [26-28,38]. It is reported that chronic ethanol exposure affects HIF [40,41]. In this study, single ethanol treatments were utilized, which did not influence cell viability. Therefore, it is thought that ethanol toxicity was not a confounding factor in the present study. Generally, in the stages of ethanol intoxication, 0.1–0.5 mg/ml blood (about 2-11 mmol/l blood) is considered subclinical. An ethanol concentration of 300 µM, which was observed to promote EPO production, is approximately 1/7-1/30 of the subclinical level and is only 0.015 promille. Therefore, it is thought that a blood concentration representative of that used experimentally is accomplished by slight alcohol intake.

In conclusion, we showed that low concentrations of ethanol promote EPO production by increasing SIRT1 in HepG2 cells and primary liver and kidney cells. The use of ethanol represents a hypoxia-independent method to promote EPO production.

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