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Erythropoietin attenuates hydrogen peroxide-induced damage of hepatocytes

Hepatositlerde oluşturulan hidrojen peroksit toksisitesinde eritropoietin'in koruyucu rolü

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Background/aims: *High levels of hydrogen peroxide (H_2O_2) are observed during inflammatory and ischemic states of the liver and usually lead to cellular dysfunction and cytotoxicity. Recently, it has been reported that erythropoietin and mitochondrial K (ATP) channel openers have a protective effect via a pharmacological preconditioning action during ischemia reperfusion injury of the liver and heart. However, it remains unclear as to whether K (ATP) channel blockers can reduce the protective effect of erythropoietin in the H_2O_2-induced injury of hepatocytes.* ***Methods:*** *To determine whether erythropoietin treatment decreases H_2O_2-induced toxicity, we used human hepatocyte cell line Hep3B for assays. Cells were pretreated with different dosages of erythropoietin (0.1-1-10-50 IU/ml) 2 h before H_2O_2 application. For determination of effects of blockage of mitochondrial K (ATP) channels during erythropoietin treatment, glibenclamide treatment was applied to the medium 2 h before H_2O_2 toxicity. Cell number, lactate dehydrogenase and caspase-3 levels were measured in erythropoietin, glibenclamide and/or H_2O_2-treated groups.* ***Results:*** *Erythropoietin treatment significantly increased cell number at the 24^{th} and 48^{th} h compared to the control group. H_2O_2 application induced apoptosis and lactate dehydrogenase release from Hep3B cells and decreased cell number. Erythropoietin prevents H_2O_2 toxicity in hepatocytes. The K channel inhibitor glibenclamide decreased the cytoproliferative and cytoprotective effect of erythropoietin during H_2O_2 toxicity of Hep3B cells.* ***Conclusions:*** *Erythropoietin treatment may be considered as a therapeutic agent during oxidative injuries of hepatocytes and its cytoprotective effect is abolished by glibenclamide.*

Key words: ATP dependent K channel, caspase-3, erythropoietin (EPO), glibenclamide, hepatocyte, hydrogen peroxide (H_2O_2) toxicity, lactate dehydrogenase (LDH)

Amaç: *Karaciğerin enflamatuar ve iskemik hasarlarında yüksek düzeyde H_2O_2 açığa çıkmakta ve genellikle hücresel disfonksiyona ve sitotoksisiteye neden olmaktadır. Son dönemlerde, eritropoietin ve mitokondrial K (ATP) kanal açıcılarının karaciğer ve kalbin iskemi reperfüzyon hasarı sırasında farmakolojik önşartlanma oluşturarak koruyucu etkiye sahip olduğu gösterilmiştir. Bununla beraber; K (ATP) kanal blokörlerinin, eritropoietinin karaciğerde oluşturulan H_2O_2 toksisitesine karşı koruyucu etkisini azaltıp azaltamayacağı henüz bilinmemektedir.* ***Yöntem:*** *Eritropoietinin H_2O_2 ile oluşturulan toksisitesindeki etkisini araştırmak amacıyla çalışmamızda insan hepatosit hücre dizisi Hep3B kullanılmıştır. Hücreler H_2O_2 uygulamasından 2 saat önce eritropoietinin farklı dozlarıyla (0.1-1-10-50 IU/ml) ön tedavi edilmiştir. Eritropoietinin uygulaması sırasında mitokondrial K (ATP) kanal blokajının etkisini görmek amacıyla glibenklamid ortama H_2O_2 toksisitesinden 2 saat önce eritropoietin ile eşzamanlı olarak eklenmiştir. Hücre sayısı, laktat dehidrogenaz ve kaspaz-3 düzeyleri eritropoietin, glibenklamid ve/veya H_2O_2 ile muamele edilmiş hücrelerde ölçülmüştür.* ***Bulgular:*** *Eritropoietin uygulaması hücre sayısını kontrol grubuna göre 24 ve 48. saatlerde belirgin bir şekilde arttırmıştır. H_2O_2 eklenmesi; hücrelerde apoptosis, hücre ölümü ve laktat dehidrogenaz salınımını arttırmıştır. Eritropoietin tedavisi; hepatositleri H_2O_2 toksisitesinden korumuştur. K kanal inhibitörü glibenklamid Hep3B lerde H_2O_2 toksisitesi sırasındaki sitoproliferatif ve sitoprotektif etkisini azaltmıştır.* ***Sonuç:*** *Eritropoietin uygulaması hepatositlerin oksidatif hasarı sırasında törapetik bir ajan olarak önerilebilir ve eritropoietinin sitoprotektif etkisi glibenklamid ile azalmaktadır.*

Anahtar kelimeler: ATP bağımlı K kanalı, kaspaz-3, eritropoietin (EPO), glibenklamid, hepatosit, hidrojen peroksit (H_2O_2) toksisitesi, laktat dehidrogenaz (LDH)

INTRODUCTION

In liver injury, hepatocytes are subjected to oxidative stress from both reactive oxidative species (ROS) generated intracellularly in response to cytokines and hepatotoxins and ROS produced extracellularly by inflammatory cells. Peroxisomal oxidases and microsomal cytochrome *P*450 enzy-

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mes are the most important sources of ROS, jointly accounting for the production of 80% of the hydrogen peroxide (H_2O_2) in the liver under normal physiological conditions (1). Both exogenously exposed and endogenously produced ROS are known to act as intermediates in apoptotic signaling. H_2O_2 is involved in numerous types of cell and tissue injury. High levels of H_2O_2 are observed during inflammatory states and usually lead to cellular dysfunction and cytotoxicity. Especially during alcohol-induced hepatotoxicity, induction of microsomal cytochrome *P*450 enzymes occurs (2). During infections and inflammation, neutrophils trigger a long-lasting oxidant stress through NADPH oxidase. Superoxide generated by NADPH oxidase dismutates to oxygen and H_2O_2, which is a highly diffusible oxidant. In addition, myeloperoxidase released from the neutrophils' azurophilic granules can generate hypochlorous acid (3). The hydroxyl and superoxide radicals generated by H_2O_2 can lead to necrotic cell injury through damaging interactions with cellular DNA, protein, or lipids, which disrupts critical cellular macromolecules and energy production (1,4).

Erythropoietin (EPO) is a hematopoietic cytokine. Decreased oxygen delivery, most often due to anemic hypoxemia, is the primary stimulus to EPO release. Typical for cytokines, EPO has multiple functions besides bone marrow. Previous studies showed that it has strong antiapoptotic and antioxidant properties (5,6). EPO activates different protein kinase signalling pathways and can increase resistance to ischemia and oxidative stress. Before ischemia reperfusion (I/R) injuries, administration of mitochondrial ATP dependent K channel opener protects neurons from oxidative damage and apoptosis (7,8). The same results were seen after a liver I/R model (9). EPO treatment during I/R damage mimics preconditioning (10). It has been shown that ATP dependent K channel activation is involved in cardioprotective effects of EPO during cardiac I/R damage (11). Previous studies demonstrated the sizeable beneficial effects of EPO in several clinical in vivo models of I/R injury (9), shock (12) and laparoscopy (10) induced damage of the liver, but direct effect of EPO treatment during H_2O_2 toxicity in hepatocytes is not yet known. In this study, we aimed to investigate whether EPO treatment is protective during H_2O_2 toxicity and its relationship with ATP dependent K channel activation.

MATERIALS AND METHODS

Cell lines, chemicals and materials:

Human hepatoma cell line Hep3B cells were obtained from the ATCC. Cells were cultured in RPMI-1640 medium (PAA, Austria), supplemented with fetal calf serum (FCS) (PAA, Austria), L-glutamine (Sigma, USA), streptomycin (Sigma, USA) and penicillin (Sigma, USA). Effect of EPO (rHuEPO – recombinant human erythropoietin- Eprex 4000 IU/0.4 ml flacon, Janssen-Cilag) treatment during H_2O_2 (Sigma, USA) toxicity was studied. Cell counts were tested by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT, Sigma, USA). For evaluation of apoptosis, caspase-3 levels were measured by a fluorometric kit (Biotium, USA). Lactate dehydrogenase (LDH) level was measured with a kit using an automatic multianalyzer (Roche; P800).

Effects of glibenclamide (Sigma, USA) during H_2O_2 toxicity were evaluated.

Cell culture and experimental protocol:

The human hepatoma cell line Hep3B was cultured in RPMI-1640 medium, supplemented with 10% v/v FCS, 2 mM L-glutamine, streptomycin (100 μg/ml) and penicillin (100 IU/ml) in a humidified atmosphere containing 5% CO_2 at 37°C. One day before the experiments, cells were seeded on 96-well microtiter plates (Nunc, Denmark) as $2X10^5$ cells/ml.

Depending on the groups, different concentrations of EPO (0.1-1-10-50 IU/ml), glibenclamide (10 μM) and/or H_2O_2 (100 μM) were added to medium. Before induction of cell death by H_2O_2, cells were pretreated with different dosages of EPO for 2 h, then H_2O_2 was applied for 2 h. Then medium was changed according to group protocols. Glibenclamide treatment was applied to medium 2 h before H_2O_2 toxicity. For determination of effects of glibenclamide during EPO treatment, we used EPO at a concentration of 50 IU/ml in the experiment.

LDH and caspase-3 levels were measured from EPO, glibenclamide and/or H_2O_2-treated groups at the 48th h. After supernatants were removed, cell surface was washed with sterile phosphate buffered saline (PBS) and cells were harvested with lysis solution, and caspase-3 levels of groups were measured from cell lysates. LDH measurement was done from both the supernatant and cell lysate.

Evaluation of cellular proliferation:

The MTT, a colorimetric assay based upon the ability of living cells to reduce 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide into formazan, was used for evaluation of the effects of H_2O_2, EPO and glibenclamide on cellular death or proliferation (2nd, 24th and 48th h).

Biochemical Determination of Cell Death

Hep3B cells were plated in 96 multi-well culture plates as $3X10^5$ cells/ml. LDH is normally present in the cytosol of hepatocytes. In response to cell damage, LDH is released from the cells. Therefore, to determine cell death, we measured secreted and intracellular LDH levels and we calculated % released LDH at the 48th h of each group. To do this, the medium was collected to measure enzyme activities. The adherent cells were lysed. Both medium and cell lysates were used for quantitative determination of LDH activity (IU/L), which was performed with an automatic multianalyzer (Roche) using kit (Roche). Released enzyme fractions for each sample were calculated as the ratio of enzyme present in the medium vs. the sum of the levels of the same enzyme in the supernatant and in the cells.

Measurement of apoptosis:

Caspase-3 levels:

The presence of apoptosis was determined by caspase-3 levels. Equal numbers of cells were used for caspase-3 level measurements. Cells were lysed with assay buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 2 mM EDTA, 2 mM EGTA, Triton X-100, 0.1%). Caspase-3 levels were measured by DEVD-R110 Fluorometric HTS Assay Kit from cell lysates. The fluorogenic substrate $(Ac\text{-}DEVD)_2$-R110 was used for this assay. It is completely hydrolyzed by the enzyme in two successive steps. Cleavage of the first DEVD peptide results in the monopeptide Ac-DEVD-R110 intermediate, which has absorption and emission wavelengths similar to those of R110 ($\lambda_{abs}/\lambda_{abs}$=496/520 nm), but has only about 10% the fluorescence of the latter. Hydrolysis of the second DEVD peptide releases the dye R110, leading to a substantial fluorescence increase.

Equal volumes of sample and caspase-3 detection buffer were added to assay plate, then incubated at 37°C for 1 h. Results were read with a fluorometer at 470 nm excitation filter and 520 nm emission filter. R110 was used for generating a standard curve to calculate amount of substrate conversion.

Statistical Analysis

Results of the experiments were analyzed by one way ANOVA, followed by a multiple comparison test using SPSS 10.0. $p < 0.05$ was accepted as statistically significant. Results are given as mean±SEM.

RESULTS

Cell proliferation and toxicity:

H_2O_2 exposure decreased living cell number immediately at the 2nd h compared to control and EPO treatment groups ($p<0.05$). At the end of the experiment (48th h), cell numbers in the H_2O_2-treated group were decreased significantly compared to the other groups ($p<0.001$). At the 48th h of EPO treatment, hepatocyte number was increased compared to H_2O_2 and control groups ($p<0.001$). There was no significant difference between cytoprotective effects of the different dosages of EPO treatment during H_2O_2 toxicity (Figure 1).

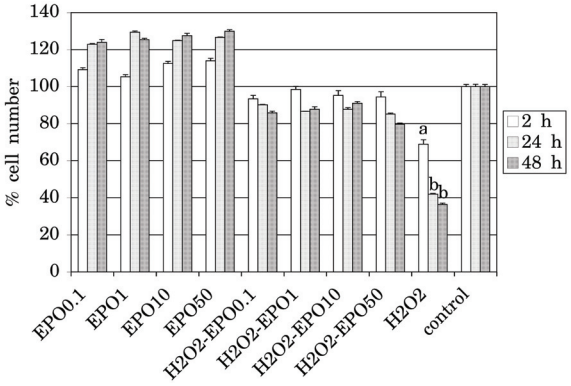

Figure 1. % Cell death was determined at 2nd, 24th and 48th h by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. H_2O_2 exposure induced prominent cell death in Hep3B hepatocytes. Data are from six independent experiments for each condition. Data are given as mean±SEM. $P<0.05$ was accepted as statistically significant.
[a]represents $p<0.05$, [b]represents $p<0.001$ difference between H_2O_2-exposed cells and the control group.

Glibenclamide diminished the proliferative and cytoprotective effect of EPO treatment ($p<0.05$) (Figure 2).

Cellular cytotoxicity of H_2O_2 was also determined by LDH release percentages at the 48th h. H_2O_2 ex-

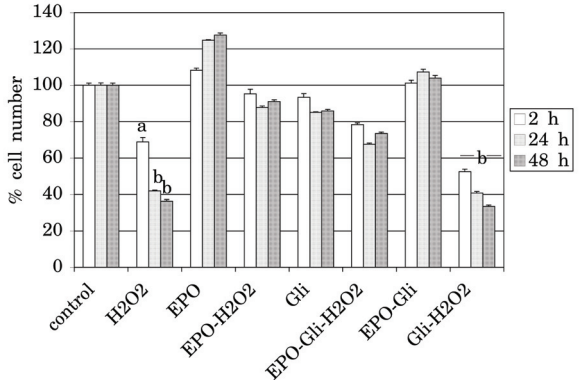

Figure 2. ATP dependent K channel blockage with glibenclamide inhibited the cytoproliferative effect of EPO. Data are given as mean±SEM. P<0.05 was accepted as statistically significant. [a]represents p<0.05, [b]represents p<0.001 difference between H_2O_2-exposed cells and the control group.

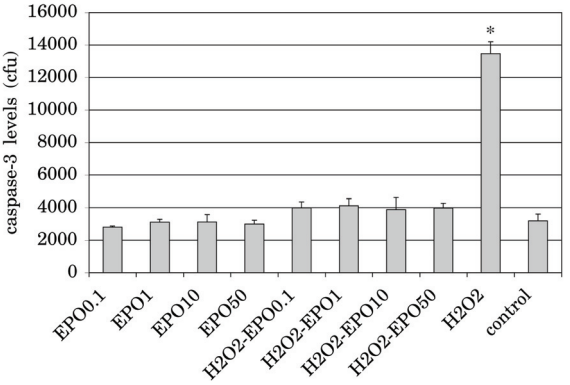

Figure 4. Caspase-3 levels: H_2O_2 toxicity caused prominent apoptosis at the 48[th] h (p<0.001). EPO treatment during H_2O_2 toxicity decreased apoptosis. Data are given as mean±SEM. *represents difference between control and H_2O_2 toxicity groups (p<0.001).

posure caused increased LDH release from Hep3B cells. EPO treatment protected hepatocytes from toxic effects of H_2O_2 (Figure 3).

Determination of apoptosis:

Caspase-3 level was used for the determination of the apoptotic effect of H_2O_2. At the 48[th] h, H_2O_2 caused apoptosis in Hep3B cells (p<0.001). EPO treatment was protective against H_2O_2 by decreasing apoptosis as measured by caspase-3 levels (Figure 4). Similar results were seen from the apoptosis assay like MTT and LDH leakage. Glibenclamide abolished the antiapoptotic effect of EPO treatment at the 48[th] h. Glibenclamide alone slightly increased apoptosis in Hep3B cells but it was not statistically significant (Figure 5).

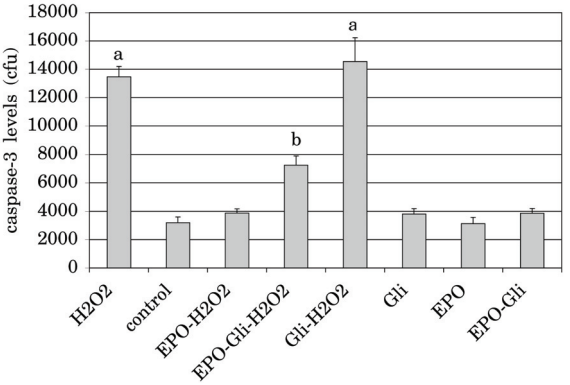

Figure 5. Caspase-3 levels: Glibenclamide, EPO, EPO+glibenclamide treatment did not cause any increase in caspase-3 levels of the hepatocytes at the 48[th] h. H_2O_2 toxicity increased caspase-3 levels compared to control (p<0.001). EPO treatment during toxicity decreased H_2O_2-induced damage (p<0.01). [a]represents difference between H_2O_2, glibenclamide-H_2O_2 and control groups(p<0.001). [b]represents difference between EPO-glibenclamide-H_2O_2 and control groups (p<0.01).

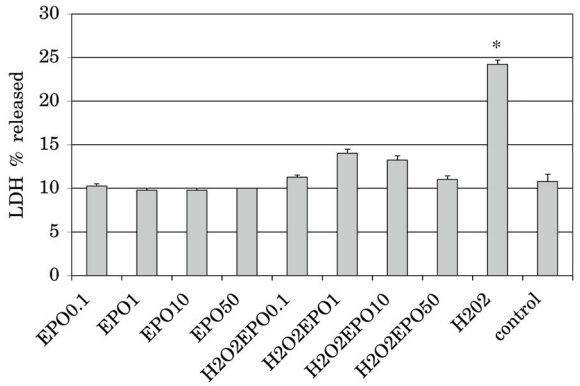

Figure 3. H_2O_2 induced cytotoxicity at the 48[th] h of the experiment determined by LDH % released to medium. H_2O_2 induced two-fold LDH release from hepatocytes at the end of the 48[th] h (p<0.01). Data are given as mean±SEM. *represents difference between control and H_2O_2 groups.

DISCUSSION

In liver diseases, ROS are involved in cell death and liver injury. During oxidative stress or ischemia, mitochondrial damage occurs; cytochrome c releases and activates downstream caspases leading to apoptosis. Application of H_2O_2 induced apoptosis and cell death in hepatocytes (1). We found increased caspase-3 level in this group. LDH is an enzyme that is normally present in the cytosol of hepatocytes. In response to cell damage (necrosis or late-stage apoptosis), LDH is released from the cells (13). Therefore, to determine cell death, we measured secreted and intracellular LDH

levels. Cell number of the H_2O_2 toxicity group was lower at the 48th h compared to 2nd and 24th h. We calculated % released LDH at the 48th h of each group. As a result of increased cellular damage, increased LDH leakage occurs from cells to the medium, as we found in the H_2O_2 toxicity group.

We applied EPO treatment before H_2O_2 toxicity. Application of EPO decreased peroxide-triggered apoptosis, LDH leakage, and cell death. Caspase-3 levels were decreased in the EPO treatment group. We found that EPO treatment has antiapoptotic and proliferative effect on hepatocytes, and this effect was independent of the dosages selected in the experimental design. EPO had a hepatoprotective effect against H_2O_2 toxicity and the nonspecific ATP dependent K channel blocker, glibenclamide, abolished this effect. Direct acute protective effects of EPO have been shown to implicate these channels. Previous studies have shown that systemic application of single-dose EPO treatment inhibits nitric oxide mediated free-radical formation in the rat liver, and reduces oxidative stress, caspase-3 levels and liver enzymes in the serum of rats after I/R injury (9-12). Our study supported these findings and we found that EPO has a direct cytoprotective and cytoproliferative effect on hepatocytes.

Information related to the role of K channels in hepatocytes is limited. The ATP-sensitive K+ channels in both sarcolemmal and mitochondrial inner membrane are the critical mediators in cellular protection of ischemic preconditioning. Activation of mitochondrial ATP dependent K^+ channels plays a significant role in the reduction of apoptosis (13,14). ATP dependent K^+ channels have significant roles in liver growth control as indicated by stimulation of DNA synthesis. It was shown that K ATP channel blockers quinidine and glibenclamide inhibited DNA synthesis both with and without hepatic growth factor stimulation in hepatocytes (15).

Opening of these channels has been related to protein kinase C activation, calcium- mediated signals and through mitogen-activated protein kinases (MAPK) activation (16,17). It is known that EPO activates protein kinase receptors and MAPK. Activation of protein kinases induces mitogenic activity in cells (18). The cytoproliferative effect of EPO is also mediated by these kinases and these effects were blocked by ATP dependent K channel blockage (19). Although we did not evaluate intracellular pathways and kinases, blockage of the cytoprotective and proliferative effects of EPO treatment by K channel blockage might be due to blockage of protein kinases.

In conclusion, these results suggest that the protective role of EPO against hepatic H_2O_2 toxicity correlated with activation of ATP dependent K channel activation.

EPO is a therapeutic drug for different liver injury models. However, further investigations are required to clarify this role, because the hepatic protective mechanisms associated with EPO and subtypes of K ATP channels are not yet clearly defined.

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