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ORIGINAL CONTRIBUTION

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Protective effects of taurine on endothelial cells impaired by high glucose and oxidized low density lipoproteins

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Abstract *Background* Endothelial dysfunction, common to diabetes and cardiovascular diseases, is an early step in the development of atherosclerosis and diabetic angiopathies. Deficiencies of taurine have been related to diabetes and cardiovascular diseases. *Aims of the study* We investigated whether taurine provides protective action against endothelial dysfunction induced by hyperglycemia and/or oxidized low density lipoproteins (oxLDL). *Methods* Quiescent human umbilical cord venous endothelial cells were exposed for 20 h to high glucose (35 mM) and/or oxLDL (60 μg/ml) alone and in presence of taurine (0.5–2.5 mg/ml). Apoptosis, caspase-3 activity, soluble(s) and cell surface expressions of vascular cellular (VCAM-1) and intercellular (ICAM-1) adhesion molecules were determined. Results are given as a percentage of the low glucose medium control. Apoptosis, VCAM-1 and ICAM-1 expressions were related to cell number. *Results* Hyperglycemia increased apoptosis to 162.5 ± 19.2%, caspase-3 activity to 153.2 ± 10.3%, cell-surface expression of VCAM-1 to 125.1 ± 5.8%, the expression of ICAM-1 to 123.7 ± 2.8% and sICAM-1 to 146.5 ± 7.9%. Taurine (0.5–2.5 mg/ml) restored apoptosis, caspase-3 activity and expressions of VCAM-1 and ICAM-1. OxLDL (60 μg/ml) increased apoptosis to 114.8 ± 3.1%; taurine (2.5 mg/ml) reduced this apoptosis to 40.5 ± 4.1%. The combination of hyperglycemia and oxLDL increased apoptosis to 211.7 ± 11.6%. This increase was normalized by taurine (2.5 mg/ml) to 97.9 ± 12.8%. *Conclusion* Taurine protects HUVECs from endothelial dysfunction induced by hyperglycemia through down-regulation of apoptosis and adhesion molecules. Counteracting the combination of oxLDL and hyperglycemia requires pharmacological concentrations of taurine.

Key words taurine – endothelium – oxLDL – diabetes – apoptosis – adhesion molecules

Introduction

Abundant evidence shows that patients with type 1 or type 2 diabetes have a high risk for several cardiovascular disorders: Coronary heart diseases (CHD), stroke, peripheral arterial diseases and congestive heart failure. Cardiovascular complications have even emerged as leading cause of diabetes–related morbidity and mortality [15]. A link between CHD and diabetes at the cellular level is the endothelial dysfunction which plays a pivotal role in the initiation

and progression of both, atherosclerosis and diabetic micro- and macroangiopathies.

Prevention of endothelial dysfunction relies primarily on the preservation of the functional and anatomical integrity of the endothelial lining of blood vessels which in turn depends upon the balance between the extent and frequency of injuries to the endothelium and the capability of the endothelium to regenerate in response to injury [36]. Hyperglycemia causes deleterious changes to endothelial cells (ECs). In vitro exposure of ECs to high ambient glucose causes a delayed replication [24], disturbed cell cycle [24], increased DNA damage [25] and increases in apoptosis [32, 42]. Moreover, high glucose modulates adhesion molecule expression and enhances the adherence of both neutrophils and monocytes to ECs [42]. Hyperglycemia in diabetes was found to be responsible for endothelial abnormalities including accelerated disappearance of capillary endothelium and weakening of intracellular junctions [23]. Furthermore, hyperglycemia is suggested to trigger a dysregulation of the vascular tone, platelet aggregation, coagulation, fibrinolysis, vascular permeability and lipoprotein oxidation through activation of advanced glycation endproduct (AGE) formation, inactivation of nitric oxide (NO) and generation of oxidative stress [18, 22]. This pro-atherogenic sequence forms the basis for an increased risk for patients with insulin-dependent diabetes mellitus (IDDM) for atherosclerotic vascular diseases.

The oxidative modifications of LDL (oxLDL) and the subsequent alterations of endothelial function are generally accepted as an important early event in the pathogenesis of atherosclerosis [21, 30]. Increased oxLDL antibody concentrations were found in patients with IDDM [30]. Endothelial function was shown to be inversely related to concentrations of oxLDL antibodies in hypercholesterolemic patients [18]. Short term incubation of ECs with LDL obtained from type 1 diabetic patients can cause deep modification in both the plasma membrane and the cytosolic compartment of ECs [33]. Also oxLDL induces apoptosis in HUVECs [8]. Thus, both hyperglycemia and oxLDL promote endothelial dysfunction. Therefore prevention of endothelial cell damage induced by these two offenders is important in preventing micro- and macrovascular diabetic complications. Ample animal studies suggest strongly that the semi-essential β-amino acid taurine has beneficial effects on lipid metabolism and hyperglycemia [9, 14]. Recently it was shown that long-term taurine supplementation reduces hyperglycemia and thereby the mortality of diabetic rats [9]. In NOD mice taurine could prevent the onset of diabetes mellitus (DM) and postnatal taurine was shown to modify the glucose-loading curves in adults [14]. Taurine exerts its cytoprotective activity by acting as an antioxidant, osmoregulator and intracellular Ca^{2+}-flux regulator [7]. It makes up over 50% of the free amino acid reserves in the myocardium and decreases cardiac neutrophil infiltration following ischemia reperfusion [7]. It up-regulates human constitutive endothelial NO-synthase, a known cytoprotector [7] and taurine pretreatment improves islet recovery after cryopreservation indicating its membrane stabilizing action [17]. It was also shown to possess insulin-like activity [6].

Insulin-dependent diabetes mellitus patients were found to possess reduced taurine concentrations both, in the plasma and in platelets [43]. A daily supplementation of 3 g taurine for seven weeks reduced triglycerides and the atherogenic index in overweight college students [12]. However, other recent clinical studies were not promising for overweight subjects [6, 37]. Most experimental evidence originates from animal studies. Studies with human ECs are rare. We therefore investigated in HUVECs the role of taurine in mitigating the deleterious effects of either high glucose or oxLDL alone or in combination considering relevant parameters for endothelial dysfunction in DM and CHD, such as apoptosis, caspase-3 activity, and the vascular cellular (VCAM-1) and intercellular (ICAM-1) adhesion molecule expression.

Material and methods

Primary cultures of HUVEC

Umbilical cords were collected in cold PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \times 7H_2O$, 1.4 mM KH_2PO_4, pH 7.4) and HUVECs were isolated and cultivated as previously described [39–41]. HUVECs were cultivated in 75 cm^2 flasks in medium 199 supplemented with 20% FCS, 10 µg/ml heparin and 30 µg/ml crude endothelial cell growth factor (ECGF).

Treatment of HUVECs

HUVECs were treated with taurine (0.5–2.5 mg/ml) in the presence of hyperglycemia (35 mM) and/or oxLDL (60 µg/ml). In case of hyperglycemia cell adhesion molecules, apoptosis and caspase-3 activities were measured; in case of oxLDL or the combination of oxLDL and hyperglycemia apoptosis was measured as described below.

Apoptosis

The number of apoptotic cells was determined by the Cell Death Detection ELISA from Roche. This photometric enzyme-immunoassay determines the

cytoplasmatic histone-associated DNA fragments (mono- and oligonucleosomes) after induced cell death in the cell lysates (apoptosis). Quiescent HUVECs were exposed to increasing concentrations of taurine (0.5–2.5 mg/ml), glucose (5.5 and 35 mM) and/or oxLDL (60 μg/ml) for 20 h. After a total of 24 h supernatants were removed. For the estimation of apoptosis cells were lysed in 200 μl lysis buffer per well. The lysate was centrifuged and the supernatants were collected and analyzed for apoptosis in a one-step sandwich ELISA according to the manufacturer's instructions. The apoptotic rate was related to the corresponding cell number.

Cell count

Cell counts were taken with the Casy 1 (Schärfe System) as described earlier [39–41]. In short, quiescent HUVEC kept in serum free medium were exposed to taurine, glucose and oxLDL as desribed above. After a total of 24 h the experiment was terminated by aspirating the medium and subjecting the cultures to sequential washes with PBS pH 7.4. Cells were detached by a mixture (1:1) of trypsin/EDTA and accutase for cell counting. Results were expressed as % of the low glucose medium "control". All experiments were performed a minimum of three times in triplicates.

Caspase-3 activity

Caspase-3 activity was determined in the cell lysates of HUVEC using the colorimetric Caspase-3 assay kit from Sigma according to the manufacturer's instructions. The assay is based on hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-p-nitroanilidine (Ac-DEVD-pNA) by caspase-3, resulting in the release of the p-nitroaniline (pNA) moiety. pNA has a high absorbance at 405 nm. The concentration of the pNA released from the substrate is calculated from a calibration curve prepared with defined pNA solutions. The caspase-3 activity per well ranged from 19.8 to 248 nmol min^{-1} ml^{-1}. The data represents four independent experiments, one performed in 24 well plates in triplicate and three in 6 well plates with single values. Results were expressed as percentage of the low glucose medium control. HUVECs were treated with taurine and glucose as described under apoptosis.

VCAM-1 and ICAM-1-expression

Cells were treated as described under apoptosis. Soluble(s) VCAM-1 and sICAM-1 were determined in the medium using an ELISA (Hoelzel Diagnostika, Germany) according to the manufacturer's instructions. Cell surface expression of VCAM-1 and ICAM-1 were determined by cell ELISA as described earlier [31]. HUVECs were incubated at room temperature with anti-human VCAM-1 (CD 106) or ICAM-1 (CD 54) monoclonal antibody (1:1,000) for 1 h in medium 199 containing 0.5% BSA and then with horseradish peroxidase-conjugated goat anti-mouse IgG at 1:1,000 dilution in the same medium for 1 h. Color formation with tetramethylbenzidin (TMB) was measured at 405 nm. Results were expressed as percentage of controls and related to cell number.

LDL-isolation and oxidation

Low density lipoproteins (d = 1.019–1.063 g/ml) were isolated as described earlier [39]. LDL-oxidation was promoted by 5 μmol/l $CuSO_4$ up to dien values between 0.9 and 1.2 for 100 μg LDL/ml [39]. Oxidation was terminated by the addition of EDTA (final concentration 1mM). Dien (λ_{234}) and peroxide (λ_{265})-values of 100 μg LDL/ml were 0.9–1.2 and 0.055–0.136 respectively and the amount of thiobarbituric acid reactive substances, determined against a standard of malondialdehyde, were 2.6–4.1 nmol. The oxidation period lasted for approximately 2 h. Quantification of LDL was performed by the determination of the protein content as described previously [39].

Statistical analysis

Data are presented as mean ± SEM. If not indicated otherwise, a minimum of three independent experiments was performed, each in triplicates with cells of a minimum of three different vessel donors. For comparison the Student's t-test was applied in case of normally distributed data, otherwise the Mann–Whitney U test was applied. Differences were considered statistically significant when $P \leq 0.05$. Data analysis was accomplished using Sigma Stat Version 1.0.

Results

Influence of taurine on the rate of apoptosis in high glucose medium

High glucose medium (35 mM) increased the rate of apoptosis to 162.5% compared to low glucose medium ($P < 0.05$). Taurine in concentrations of 0.5, 1.5 and 2.5 mg/ml reduced apoptosis again significantly to values which were comparable to those in low glucose medium (Fig. 1a).

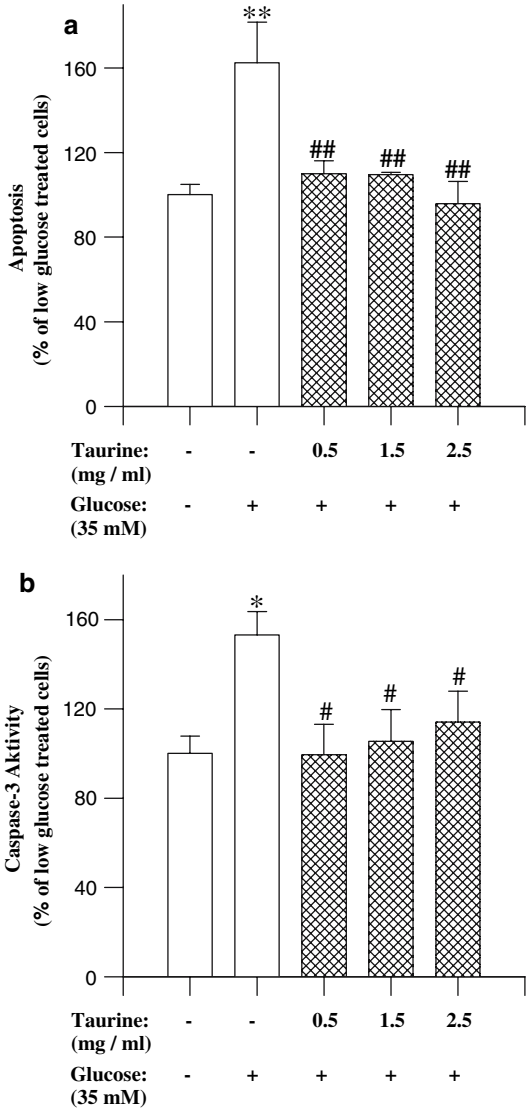

Fig. 1 Effect of different concentrations (0.5, 1.5, 2.5 mg/ml) of taurine (20 h) on HUVEC in medium containing high (35 mM) glucose with respect to (**a**) apoptosis and (**b**) caspase-3 activity (measured as detailed in material and methods). Data represents the mean ± SEM of at least three independent experiments each in triplicate and is presented as a percentage of the low (5.5 mM) glucose medium control. Apoptotic rates were related to the corresponding cell number. *: $P < 0.05$; **: $P < 0.01$, (versus low glucose medium control), #: $P < 0.05$; **##**: $P < 0.01$ (versus high glucose medium)

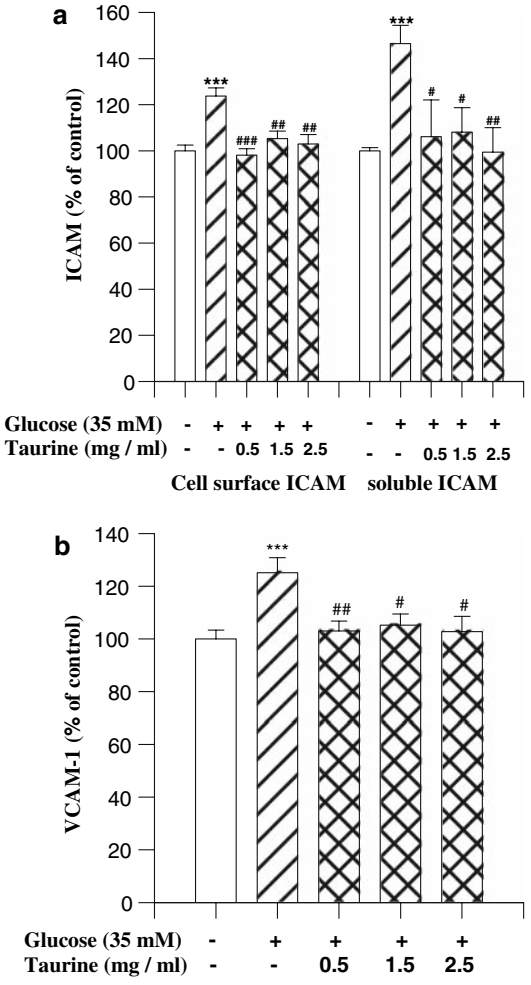

Fig. 2 Effect of different concentrations (0.5, 1.5, 2.5 mg/ml) of taurine (20 h) on HUVEC in medium containing high (35 mM) glucose with respect to (**a**) soluble(s) and cell surface ICAM-1 (**b**) cell surface VCAM-1. Data represents the mean ± SEM of at least three independent experiments each in triplicate and is presented as a percentage of the low (5.5 mM) glucose medium control. Adhesion molecule expressions were related to the corresponding cell number. ***: $P < 0.001$ (versus low glucose medium control), #: $P < 0.05$, ##: $P < 0.01$, ###: $P < 0.001$ (versus high glucose medium)

Influence of taurine on caspase-3 activity in high glucose medium

To further elucidate the mechanism of action of taurine in the process of apoptosis, we estimated the caspase-3 activity in HUVECs in high glucose medium. In the presence of high glucose concentrations for 20 h the caspase-3 activity in HUVECs increased significantly to 153.2% ($P < 0.05$). This increased caspase-3 activity was reduced significantly in the presence of taurine (0.5–2.5 mg/ml) (Fig. 1b).

Influence of taurine on cell surface expression of ICAM-1 and VCAM-1 and their soluble forms in high glucose medium

In the presence of high glucose concentrations the cell surface expression of ICAM-1 and VCAM-1 increased significantly to 123.7% ($P < 0.01$) and 125.1% ($P < 0.01$) respectively. These enhanced expressions could be reversed in the presence of taurine in concentrations of 0.5–2.5 mg/ml (Fig. 2a, b).

Soluble(s) ICAM-1 increased in the high glucose medium to 146.5% ($P < 0.01$). The addition of increasing concentrations of taurine (0.5–2.5 mg/ml) reduced this expression to normal values (Fig. 2b). sVCAM-1 was not detected in the medium.

Influence of taurine on the apoptosis in the presence of oxLDL in low and high glucose medium

OxLDL (60 µg/ml) increased the rate of apoptosis in HUVECs in low and high glucose to 114.8 and 211.7% respectively (Fig. 3a). In low glucose medium the addition of taurine (2.5 mg/ml) did reduce the rate of apoptosis to 40.5% (Fig. 3). In the presence of the combination of high glucose and oxLDL, taurine (2.5 mg/ml) reduced the apoptotic rate to 97.9%. Lower concentrations of taurine were not effective.

Discussion

The prevalence of type 2 diabetes mellitus is increasing at an epidemic growth rate [6] and CHDs have the highest mortality rate worldwide. Therefore nutritional factors with preventive action are of high interest. According to animal studies taurine is proposed to be a preventive factor for both developments

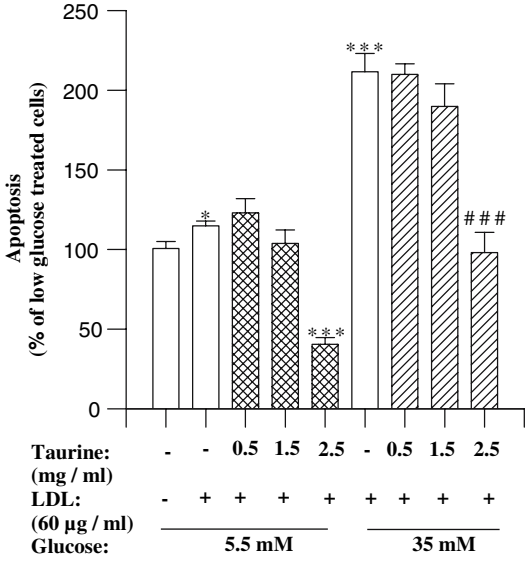

Fig. 3 Effect of different concentrations (0.5, 1.5, 2.5 mg/ml) of taurine (20 h) on HUVEC in absence or presence of 60 µg/ml oxidized LDL in medium containing low (5.5 mM) and high (35 mM) glucose with respect to apoptosis. Data represents the mean ± SEM of at least three independent experiments each in triplicate and is presented as a percentage of the low glucose medium control. Apoptotic rates were related to the corresponding cell number. *: $P < 0.05$, ***: $P < 0.001$ (versus low glucose medium control), ###: $P < 0.001$ (versus high glucose medium)

[9, 13, 27], endothelial dysfunction and pro-atherosclerotic processes. But studies in human cells are rare. We report here that taurine (0.5–2.5 mg/ml) can normalize the cell surface expression of VCAM-1 and ICAM-1 and apoptotic rates in human ECs impaired by hyperglycemia and also the apoptosis impaired by oxLDL or the combination of oxLDL and hyperglycemia. Thus, taurine can influence in vitro relevant mechanisms for the prevention of endothelial dysfunction, which precedes and predicts clinical vascular disease in human atherogenesis and should be considered a target for therapy [2].

We observed an increase in apoptosis as early as 20 h after incubation with high glucose. This "early effect" compared to other investigators [10, 42], who observed this effect after 48 h or 14 days, may be due to the higher glucose concentration (35 mmol/l compared to 30 mmol/l) which can be found in decompensated diabetes, or be a result of the use of non-synchronized ECs or both. But differences of ECs in the sensitivity to high glucose levels have also been observed. Baumgartner-Parzer et al. demonstrated that primary cultures of HUVECS were susceptible for the induction of apoptosis by high glucose but certain established cell lines (K562, P815, YT) were not [3].

Hyperglycemia increases in HUVECs oxidative stress through the overproduction of reactive oxygen species (ROS) at the mitochondrial transport chain levels [32]. ROS can subsequently activate the nuclear transcription factor-κB [10], but also the ROS-dependent matrix metalloproteinases (MMP)-2 (33 mM glucose) to induce apoptosis [19]. Taurine is a well known antioxidant and may suppress the production of ROS and subsequently apoptosis as shown in our experiments. Wu et al. demonstrated earlier that apoptosis induced in HUVECs by 48 h of hyperglycemia is attenuated by ROS-inhibition and $[Ca^{2+}]_i$-stabilization [42]. Observations that taurine reduces the cell damage associated with the ischemia-reperfusion phenomena [28] support the assumption of an antioxidant action of taurine to suppress apoptosis.

It was also reported that taurine increases the Ca^{2+}-influx into HUVECs [1, 42]. This influx may additionally contribute to the reduction of apoptosis. But our findings that taurine reduces the hyperglycemia-induced caspase-3 activity may point towards another mechanism of action. Taurine, the degradation product of the sulfur-containing amino acids cystein and methionin, may influence the activity of cysteine-requiring aspartate proteases (caspases). However, the down-regulation of caspase-3 activity may simply indicate the reduction of apoptotic activity without any direct relation to taurine.

Another mechanism of action of taurine is that of an osmoregulator. Porcine pulmonary arterial ECs

can accumulate taurine during hypertonic stress, enabling them to grow well [29]. In cerebellar neurons in culture the increase of taurine efflux closely followed the occurrence of apoptotic death markers such as caspase induction and chromatin condensation [20]. But for the glucose-induced apoptosis the control of the osmolytic situation seems to be less important, since mannose in comparable concentrations did not induce apoptosis in HUVECs [42]. Our observation that mean cell volumes of HUVECs were not significantly different in high glucose medium supports this assumption (data not shown).

The inhibition of apoptosis by taurine has been observed in several cell types: in ECs, in human neutrophils [35], rat hepatocytes [34] and pancreas cells, the latter being of special interest to diabetes. Here taurine acted via IGF-II and Fas [5]. Thus, taurine appears to perform a modulatory function on cell regeneration through apoptosis. This modulation could be of relevance in vivo to support the regeneration of the endothelium.

Apoptotic ECs become proadhesive for nonactivated platelets, neutrophils and monocytes, thus exerting a procoagulant activity [4]. We can confirm that HUVECs impaired by hyperglycemia have not only an increased rate of apoptosis, but also increase their cell surface expression of ICAM-1 and VCAM-1 as well as their release of sICAM. We could further demonstrate that taurine can reduce the expression of these adhesion molecules on the cell surface in human ECs. This expression is known to be regulated by reduction/oxidation (redox)-sensitive transcription factors, in particular NF-κB [26]. Thus, taurine may act here as already proposed for apoptosis via NF-κB.

The relevance of the down-regulation of VCAM-1 and ICAM-1 for the in vivo situation could be that a critical step in the inflammatory cascade—the adhesion of leucocytes to the vascular endothelium via the high-affinity interaction between ICAM-1 and its ligand CD11b/18—is interrupted by taurine. Indeed, a recent report on a rat model demonstrates that the pre-supplementation of pharmacological doses of taurine reduces the interaction of leucocytes with the endothelium as well as endothelial cell apoptosis and decreases ICAM-1 expression in the post-capillary venular ECs. These findings were associated with a significant reduction in central blood pressure and cardiac injury as assessed by troponin T in these rats [7]. These results point towards taurine as being beneficial in preventing critical and initial steps in microvascular injuries. However, further clinical studies are required.

Another stressor, responsible for microvascular injuries, is oxLDL. Here we can confirm earlier findings that oxLDL promotes apoptosis [8]. It also inhibits DNA-synthesis and cell proliferation of ECs [39]. An analysis of the manner in which oxLDL exerts this action in HUVECs revealed that a time-dependent accumulation of 7-ketocholesterol and a progressive oxidative modification of peroxireduxin 2 occurs, together with the suppression of cell proliferation [21]. Proteomic analysis revealed an altered expression of nucleophosmin, stathmin and nucleolin proteins involved in cell proliferation under these oxidative stress conditions [21]. Here we can show that taurine (2.5 mg/l) can reverse apoptosis induced by 60 μg/ml oxLDL. It is interesting to note that even though oxLDL induced only a small increase of apoptosis, taurine reduced the same to a remarkable extent (to 40%). This speaks for a high endogenous rate of apoptosis of these fastidious cells, likely due to the serum-free experimental conditions. But also the oxysterol content of oxLDL plays a critical role in the propagation of apoptosis [16]. A very recent report on HUVECS showed that taurine in response to oxLDL (100 μg/ml for 24 h) attenuated increases of lactate dehydrogenase as well as asymetric dimethylarginine, a major endogenous nictric oxide (NO) synthase inhibitor, TNF-α and malondialdehyd [38]. Whether these actions are all mediated by the antioxidative activity of taurine requires further investigation.

The results discussed so far suggest a preventive role of taurine for DM and CHD. However, the recent clinical studies in humans are controversial. Even though taurine supplementation (3 g/day) was shown to have a beneficial effect on the lipid metabolism in overweight or obese subjects [43], other clinical studies demonstrate that taurine had no effect on insulin secretion and action or serum lipid levels in overweight men with a genetic predisposition for type 2 diabetes mellitus [6]. Also an effect of taurine on platelet aggregation in men with a predisposition to type 2 diabetes mellitus was not observed [37]. However, taurine supplemention (1.5 g/day) comparable to the daily intake of 100 g of fish had a beneficial impact on macrovascular endothelial function in the form of normalizing NO and endothelin release as well as on the endothelial NO-synthase expression in smokers [11].

A look into the effective concentrations of taurine in our in vitro experiments reveals that the effects of hyperglycemia could be influenced by even 0.5 mg/ml, whereas counteracting the effects of oxLDL alone or in combination with hyperglycemia required 2.5 mg/ml of taurine. Administration of 3 g/day in humans, however, will lead to an effective concentration lower than 2.5 mg/ml. Thus, considering the concentrations necessary to counteract both stressors in vitro, it is unlikely that taurine alone will block or reverse an established endothelial dysfunction in vivo.

In summary, taurine has a protective impact on parameters of endothelial dysfunction in human ECs

deranged by hyperglycemia through modulating apoptosis, caspase-3 activity and the expression of VCAM-1 and ICAM-1. If oxLDL in combination with hyperglycemia stresses ECs, pharmacological concentrations are necessary to influence apoptosis in vitro.

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