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4-Hydroxy tempol-induced impairment of mitochondrial function and augmentation of glucose transport in vascular endothelial and smooth muscle cells

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Abstract

The water-soluble and cell permeable nitroxide derivative 4-hydroxy tempol (TPL) has been shown to reduce or ameliorate oxidative stress-induced dysfunction and damage in vascular endothelial cells. We studied the effects of TPL on glucose transport and metabolism in bovine aortic endothelial (VEC) and smooth muscle cells (VSMC) under normal and high glucose conditions. Normally, these cells operate an autoregulatory protective mechanism that limits the rate of glucose transport under hyperglycemic conditions by decreasing the cell content of their typical glucose transporter GLUT-1 mRNA and protein as well as its plasma membrane abundance. TPL augmented the rate of glucose transport both under normo- and hyperglycemic conditions by increasing GLUT-1 mRNA and protein content and its plasma membrane abundance in both types of cells, leading to an increased flux of glucose into the cells. These effects were found related to ROS-generating and oxidant activities of TPL and to a decreased rate of mitochondrial ATP production under both normo- and hyperglycemic conditions. Since impaired mitochondrial functions, and in particular decreased rate of ATP production, augment the expression of GLUT-1 protein and glucose transport and metabolism, we suggest that the stimulatory effects of TPL in vascular cells results from its unfavorable interactions in the mitochondrion. It is therefore suggested that effects of TPL in cells of cardiovascular system be evaluated in parallel to its adverse effects on glucose and energy metabolism.

Keywords: Diabetes; Glucose transport; Free radicals; 4-Hydroxy tempol; Vascular cells

1. Introduction

Impaired endothelium-dependent vasodilation is a serious vascular complication developed in hyperglycemic diabetic human individuals and animal models [1–6]. It has been associated with an increased production of reactive oxygen species (ROS) [7–9] through non-enzymatic glucose autooxidation coupled to protein glycation [10–13]. Also, increased expression and activity of NADP oxidase is associated with an augmented superoxide production in diabetes, atherosclerosis, hypertension and heart failure [14–17]. In addition, the activation of the polyol pathway and stimulation of the PKC isoforms have been linked to increased ROS production in diabetes [18,19].

Impaired endothelial nitric oxide synthase (eNOS) function in diabetic vessels [20–22] was associated with superoxide-dependent oxidation of tetrahydrobiopterine, an essential cofactor of eNOS, functional uncoupling of the

Abbreviations: AG, aminoguanidine; dGlc, 2-deoxy-D-glucose; DMEM, Dulbecco's modified Eagle's medium; DNP, 2,4-dinitrophenol; ESC, esculetin; GLUT-1, glucose transporter 1; GLUT-4, glucose transporter 4; H_2DCF, 2′7′-dichlorodihydrofluoresceindiacetate; HETE, hydroxyeicosatetraenoic acid; LO, lipoxygenase; NAC, *N*-acetyl-L-cysteine; PBS, phosphate buffered saline; ROS, reactive oxygen species; RT–PCR, reverse transcriptase–PCR; TPL, 4-hydroxy tempol; VEC, vascular endothelial cells; VSMC, vascular smooth muscle cells

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enzyme and generation of superoxide anions rather than NO radicals [17,23]. Thus, it has been suggested that antioxidants, especially those with superoxide dismutase properties, such as nitroxides, may reverse and correct ROS-dependent endothelial cell dysfunction [24].

Nitroxides are water soluble, stable, cell permeable and considered non-immunogenic antioxidants [25]. Nitroxide-dependent elimination or reduction of oxidative damage has been observed both in in vitro and in vivo model systems [26]. Of interest are studies on such effects of 4-hydroxy tempol (TPL, 4-hydroxy-2,2,6,6-tetraethylpiperidine-*N*-oxyl) on VEC function in diabetes and other pathological conditions. Specifically, TPL accelerated superoxide dismutation and increased the amount of NO released from bovine aortic endothelial cells [27]. It restored acetylcholine-dependent vasodilation of renal afferent arterioles from alloxan-diabetic rabbits [4] and of aortic rings isolated from streptozotocin-diabetic rats [2]. Acute hyperglycemia in dogs increased ROS production in vivo and reduced both global and local oscillatory wall shear stress in intact coronary arteries; both parameters were normalized by an intravenous infusion of TPL [28].

TPL has also been found useful in treating other oxidative stress-related vascular dysfunctions: Angiotensin II-induced hypertension in rats was prevented by orally administered TPL [29]. Chronic treatment with TPL prevented vascular remodeling and progression of hypertension in spontaneously hypertensive rats [30,31]. It also corrected systolic blood pressure and responsiveness to acetylcholine while reducing plasma levels of advanced oxidative protein products in nephrectomized rats [32]. Interestingly, VSMC also respond to diabetic conditions by increasing the production of superoxide anions [33]; but less is known about TPL effects in these cells.

Several molecular mechanisms for the antioxidant activity of TPL and other nitroxide derivatives have been proposed: (i) inhibition of the iron-driven Fenton reaction from superoxide anions or hydrogen peroxide by oxidizing transition metal ions, such as iron [26,34]; (ii) nitroxides being genuine SOD mimics that catalyze the dismutation of superoxide anions [35]; and (iii) a direct superoxide anion scavenging activity of nitroxides has also been proposed [36]. On the other hand, there are reports that TPL may also act as a pro-oxidant by oxidizing reduced transition metals and potentiating cellular damage by producing ROS [37]. Other potential harmful interactions of nitroxides with transition metal ions in catalytic centers of enzyme complexes, which may alter essential cell functions, received less attention. Such interactions with the respiratory chain complexes and/or 12-lipoxygenase (12-LO) can alter glucose transport and metabolism by impairing mitochondrial ATP production and/or 12-LO-dependent 12-hydroxyeicosatetraenoic acid (12-HETE) synthesis, respectively [38,39].

Since TPL and other nitroxide derivatives may be developed for treatment of endothelial dysfunction in diabetes and other cardiovascular dysfunctions we studied the effects of TPL on the glucose transport system and energy metabolism in bovine aortic endothelial and smooth muscle primary cell cultures.

2. Materials and methods

2.1. Compounds

DMEM and sera were from Kibbutz Beth-Haemek Biological Industries. ([U-^{14}C]sucrose (500 mCi/mmol)) and [α-^{32}P]dCTP (3000 Ci/mmol) were from Amersham Pharmacia Biotech. American Radiolabeled Chemicals supplied 2-[1,2-^{3}H(N)]deoxy-D-glucose (60 Ci/mmol; [^{3}H]dGlc). ATP (magnesium salt), bakers yeast hexokinase, bovine serum albumin, 2-deoxy-D-glucose (dGlc), digitonin, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), D-glucose, glucose-6-phosphate dehydrogenase (from *Leuconostoc mesenteroides*), glutamic acid, malic acid, NADP (sodium salt), *N*-acetyl-L-cysteine, Tri Reagent™ and streptavidin-agarose beads were purchased from Sigma Chemicals. TPL was from Aldrich Chem. Co. Sulphosuccinimidyl 6-(biotinamido)-biotin (NHS-LC-biotin) was from Pierce. 2′7′-Dichlorodihydrofluoresceindiacetate (H_2DCF) was from Molecular Probes. 5-, 12- and 15-HETE were purchased from Cayman Chemicals. Enzymes, buffers and reagents for RT–PCR were purchased from Promega Corp. All other chemicals, reagents and solvents were reagent-, molecular biology- or HPLC-grade.

2.2. Vascular cell cultures

Primary cultures of bovine aortic endothelial and smooth muscle cells were prepared and characterized morphologically and immunohistochemically as described previously [40].

2.3. Hexose uptake assay

The [^{3}H]dGlc uptake assay in VEC and VSMC was performed as described [40]. The uptake data were calculated on the basis of cell number determined by counting the cells in a haemacytometer following their detachment by trypsin treatment.

2.4. Measurement of total and plasma membrane-associated GLUT-1 and GLUT-4

Western blot analyses of total and cell surface-localized, biotinylated GLUT-1 (using a rabbit antiserum prepared against the human erythrocyte transporter, courtesy of Dr. H.-G. Joost, German Institute of Human Nutrition,

Bergholz-Rehbrücke, Germany) and GLUT-4 (using a rabbit antiserum against GLUT-4 C-terminal) were performed as previously described [38].

2.5. RNA isolation, cDNA synthesis and competitive PCR for quantification of GLUT-1 mRNA

Total RNA was extracted from 1×10^6 to 2×10^6 cells using Tri Reagent™ according to the manufacturer's protocol. GLUT-1 cDNA synthesis and competitive RT–PCR of GLUT-1 were performed as described [38].

2.6. Mitochondrial ATP production

The rate of mitochondrial ATP synthesis from glutamic acid and malic acid, which enter the respiratory chain as substrates for Complex I, was measured in digitonin-permeabilized VEC and VSMC, according to Wanders et al. [41]. Confluent cell cultures were washed with PBS and incubated for 10 min at 37 °C with a buffer containing 12 mM HEPES (pH 7.4), 140 mM KCl, 4.6 mM KH_2PO_4, 0.1 mM EDTA, 0.5 mg/ml BSA, 0.7 mM ADP, 10 mM glutamic acid, 0.5 mM malic acid and 13 μg/ml digitonin. The incubation was terminated with perchloric acid [final volume 3% (v/v)], neutralization to pH 6.5–7.0 with K_2CO_3 and centrifugation at $800 \times g_{av}$ for 10 min at 4 °C. The supernatants were used to determine the amount of ATP using the hexokinase/glucose-6-phosphate dehydrogenase coupled reaction. The reaction mixture (200 μl) contained 20 μl cell lysate or standard ATP solution, 40 mM HEPES (pH 7.4), 10 mM glucose, 0.2 mM $NADP^+$, 10 mM $MgCl_2$, 0.1 U hexokinase and 0.2 U glucose-6-phosphate dehydrogenase, and the assay was carried as described [42]. Protein content in the cell lysates was determined according to Bradford [43].

2.7. Fluorescent determination of ROS

VEC and VSMC, which were maintained at 5.5 or 23.0 mM glucose in the absence or presence of TPL, were washed and incubated with modified Gey's buffer (10 mM HEPES, pH 7.4, 145 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgSO_4$, 1 mM Na_2HPO_4, TPL and 5.5 or 23.0 mM glucose) and loaded with 10 μM H_2DCF for 15 min in the same buffer and washed again. The cells were then exposed to TPL in Gey's buffer containing 5.0 or 23.0 mM glucose, and the fluorescence intensity was measured 15 min later in a FLUOstar Galaxy plate reader fluorometer (excitation at 485 nm; emission at 530 nm).

2.8. Protein oxidation detection

Oxidation of proteins by oxygen free radicals results in an introduction of carbonyl groups into protein side chains. The OxyBlot™ oxidation detection kit (Chemicon International) was used to determine by Western blotting the extent of protein carbonylation in vascular cell lysates according to the manufacturer's protocol.

2.9. Extraction of HETEs from cell culture medium and HPLC analysis

Aliquots of cell culture media were collected, extracted and analyzed for 5-, 12- and 15-HETE by HPLC as described previously [38].

2.10. Cell viability assay

VEC and VSMC, grown and treated in 6-well plates, were washed with PBS at room temperature and incubated with the same buffer supplemented with of 0.5 mM MTT for 30 min at 37 °C. The cell were then washed three times with PBS at room temperature and incubated for 10 min with 1 ml DMSO. Aliquots were placed in 96-well plates and the intensity of cell-associated MTT was measured in a plate-reader spectrophotometer at 540 nm. Background absorbance of medium-treated aliquots was subtracted.

2.11. Glucose and lactic acid determination

The levels of D-glucose and L-lactic acid in culture medium samples were determined with enzyme-based colorimetric kits (#315-100 and #735-10, respectively) from Sigma Diagnostics® (St. Louis, MO).

2.12. Statistical analysis

Statistical analysis was done using Mann–Whitney test.

3. Results

3.1. TPL-dependent stimulation of glucose transport and metabolism in VEC and VSMC

VEC and VSMC cultures were preconditioned at 5.5 and 23.0 mM glucose for 48 h (medium was changed once at 24 h). High glucose-dependent downregulation of glucose transport was apparent in these cells, as was shown before [38]: The rate of dGlc uptake in VEC maintained at 5.5 mM was 52 ± 2 pmol dGlc/10^6 cells/min and was reduced by 39% to 32 ± 4 pmol dGlc/10^6 cells/min under the hyperglycemic conditions (Fig. 1A, time zero). Similarly, the rate of glucose transport decreased in VSMC by 49%, from 278 ± 6 pmol dGlc/10^6 cells/min to 142 ± 10 pmol dGlc/10^6 cells/min, at 5.5 and 23.0 mM glucose, respectively (Fig. 1B, time zero).

TPL (5.0 mM) increased the rate of dGlc uptake in VEC and VSMC in a time-dependent manner under both glycemic conditions. Its effect in VEC exposed to 23.0 mM was larger than at 5.5 mM glucose (225 and 170% increase, respectively). Half maximal and maximal effects were

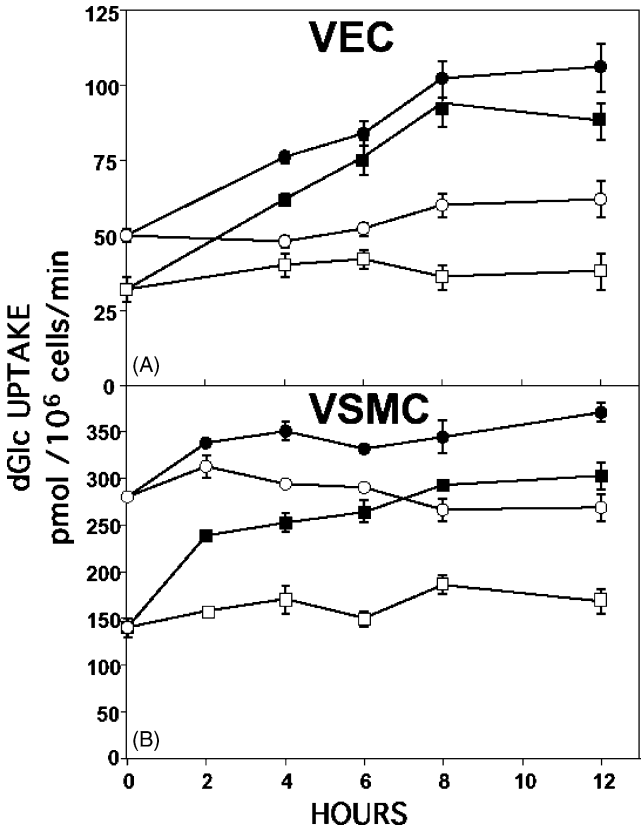

Fig. 1. Time-course of TPL-dependent stimulation of hexose transport in VEC and VSMC. VEC (A) and VSMC confluent cultures (B) were preincubated with 5.5 (circle symbols) or 23.0 mM glucose (square symbols) for 48 h. TPL (5.0 mM, ■, ●) or vehicle (DMEM: □, ○) were present during the last 12 h of incubation. [^{3}H]dGlc uptake assay was performed at the indicated times following the addition of TPL. Means ± S.E.M., $n = 3$.

observed at 5 and 8 h after its addition (Fig. 1A). Similar observations were made in TPL-treated VSMC: 181% versus 139% increase of hexose transport at 23.0 and 5.5 mM glucose, respectively. Half maximal and maximal effects were observed at 2 and 8 h, respectively (Fig. 1B). The effect of TPL was reversible: Wash-out experiments of TPL-treated VEC and VSMC showed that the rate of dGlc uptake returned to the respective control rates that were measured in untreated VEC and VSMC within 24 h (data not shown).

Dose–response analyses showed that 2.0 and 5.0 mM TPL produced half maximal and maximal effects, respectively, in both types of cells (data not shown). Therefore, 5.0 mM TPL was used in this study. The viability of the cells was not affected at this concentration of TPL, up to a 12-h incubation period, as shown by the MTT assay (Table 1).

The effect of TPL on glucose consumption and lactic acid production during a 10-h exposure period was also determined. Untreated VEC maintained at 23.0 mM glucose consumed 0.54 ± 0.04 μmol D-glucose/10^6 cells (mean ± S.E.M., $n = 3$) and released 0.89 ± 0.11 μmol L-lactic acid/10^6 cells. The corresponding values were

Table 1
Vascular cell viability is not affected by TPL

Cell type	Glucose (mM)	
	5.5	23.0
VEC	94.8 ± 5.6	95.8 ± 4.8
VSMC	96.4 ± 4.4	99 ± 2.3

VEC and VSMC were treated with 5 mM TPL for 10 h as described in the legend to Fig. 1. At the end of the incubation period the cells were taken to the MTT-cell viability assay as described in Section 2. The OD values for VEC and VSMC maintained at 5.5 and 23.0 mM glucose, taken as 100%, were 0.39 ± 0.01 and 0.33 ± 0.02, respectively (VEC); 0.42 ± 0.05 and 0.47 ± 0.02, respectively (VSMC). Mean ± S.E.M. ($n = 3$).

elevated in the presence of TPL to 2.02 ± 0.28 μmol/10^6 cells and 3.46 ± 0.27 μmol/10^6 cells. Untreated VSMC maintained at 23.0 mM glucose consumed 1.57 ± 0.13 μmol D-glucose/10^6 cells and released 2.66 ± 0.31 μmol L-lactic acid/10^6 cells, while in the presence of TPL the corresponding values increased to 5.91 ± 0.68 μmol/10^6 cells and 10.18 ± 1.09 μmol/10^6 cells. These data show that TPL-stimulated glucose transport is coupled to an augmented glycolytic flux in VEC and VSMC and also confirm previous observations that the rates of glucose transport and glycolysis in VEC are considerably lower than in VSMC [38,40].

Other antioxidants were also tested: Vitamin C (0.1–2.0 mM), the Vitamin E water soluble derivative, trolox (0.01–0.5 mM) and *N*-acetyl-L-cysteine (0.1–20.0 mM; NAC) were added for 1–24 h to VEC and VSMC cultures that were maintained at 5.5 or 23.0 mM glucose. None of these antioxidants altered the basal rate of hexose transport nor did they reverse high glucose-induced downregulation of glucose transport system. For instance, the rate of hexose uptake in VEC and VSM, which were pre-exposed to 23.0 mM glucose for 48 h, were 45 ± 2 pmol/10^6 cells/min and 122 ± 12 pmol/10^6 cells/min, respectively. The antioxidants were added (at the highest concentrations specified above) for the last 12 h of incubation. The rates of hexose uptake in cells treated with Vitamin C, trolox and NAC were not altered significantly and were 41 ± 2 pmol dGlc/10^6 cell/min, 47 ± 2 pmol dGlc/10^6 cell/min and 38 ± 6 pmol dGlc/10^6 cell/min respectively, for VEC, and 119 ± 7 pmol/10^6 cells/min, 127 ± 15 pmol/10^6 cells/min and 103 ± 10 pmol/10^6 cells/min, respectively, for VSMC. In addition, aminoguanidine (AG), an inhibitor of the formation of advanced glycosylation end products [44], did not affect the rate of hexose transport in VEC and VSMC maintained under normo- or hyperglycemic conditions. For instance, the rate of hexose transport in VEC and VSMC exposed to 23.0 mM were 38 ± 3 pmol dGlc/10^6 cells/min and 121 ± 9 pmol dGlc/10^6 cells/min, respectively. The respective rates of hexose uptake in the presence of 1 mM AG during the last 12 h of incubation were 41 ± 3 pmol dGlc/10^6 cells/min, for VEC, and 122 ± 8 pmol dGlc/10^6 cells/min, for VSMC. The lack of effect of these antioxidants and AG, but not of TPL,

suggest that the latter utilizes a distinct cellular mechanism that is not shared by the other antioxidants.

3.2. Effects of TPL, Vitamin C, trolox, N-acetyl-L-cysteine and aminoguanidine on protein carbonylation in VEC and VSMC

Since the antioxidants tested and aminoguanidine did not affect the glucose transport system in the vascular cells, while TPL did, it was important to determine whether they and TPL exerted antioxidant activities in vascular cells exposed to high glucose levels. Fig. 2A shows a significantly higher content of carbonylated proteins in VEC and VSMC exposed to 33.0 mM glucose for 3 days in comparison to cells maintained at 5.5 mM glucose. Also, the profiles of carbonylated proteins were similar in VEC and VSMC that were incubated with 5.5 mM D-glucose or with 5.5 and 28.0 mM L-glucose. These findings indicate that the increased osmotic pressure under the hyperglycemic conditions had no effect on the extent of protein carbonylation.

Equally important, Fig. 1A shows that TPL exerted prooxidant activity and increased the level protein carbonylation above the control levels of cells exposed to either

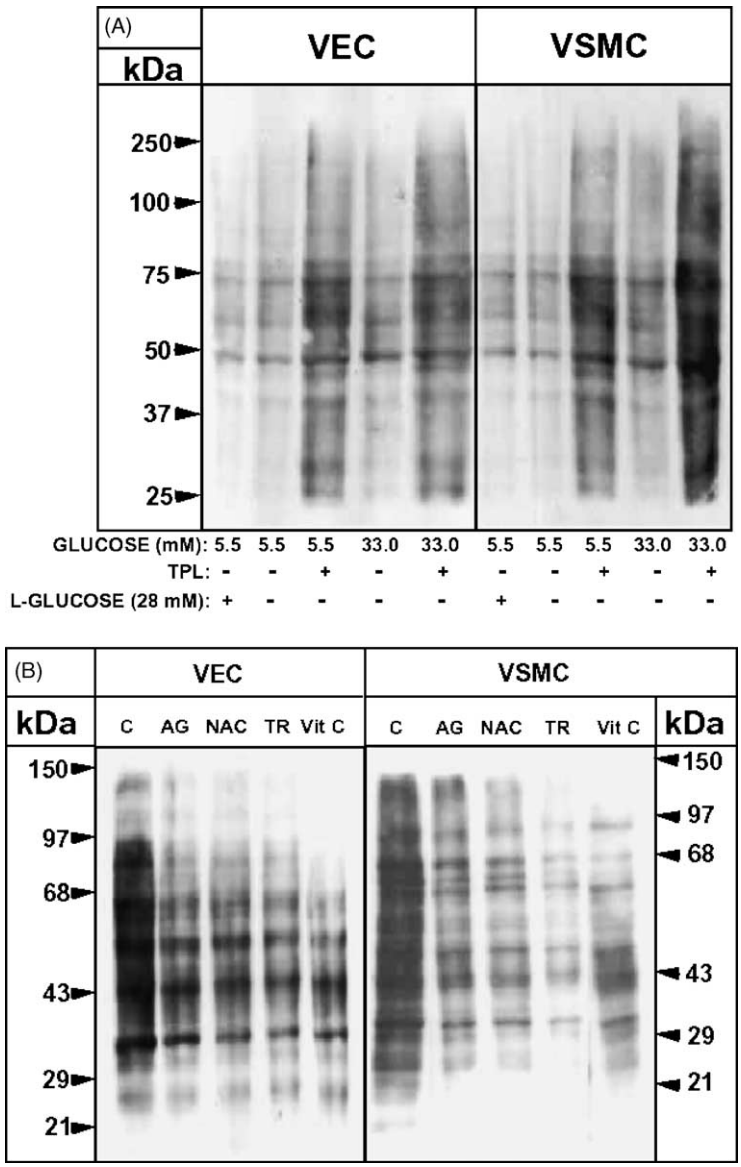

Fig. 2. Effects of high glucose level, TPL, aminoguanidine, *N*-acetyl-L-cysteine, trolox and Vitamin C on protein carbonylation in VEC and VSMC. (A) Confluent cell cultures were exposed to 5.5 or 33.0 mM glucose in the absence or presence of 5.0 mM TPL for 3 days. Control cell were also incubated with 5.5 mM glucose and 28.0 mM L-glucose. The cells were then lysed and protein-bound carbonyl groups were derivatized with 2,4-dinitiphenylhydrazine according to the OxyBlot™ oxidation detection kit instructions. Protein samples were separated by SDS–PAGE and carbonylated proteins were detected with rabbit anti-dinitrophenylhyrazone polyclonal antibody. (B) VEC and VSMC were exposed for 3 days to 33.0 mM in the absence (C) or presence of 1.0 mM aminoguanidine (AG), 5.0 mM *N*-acetyl-L-cysteine (NAC), 0.5 mM trolox (TR) or 1.0 mM Vitamin C (Vit C) and processed as described above.

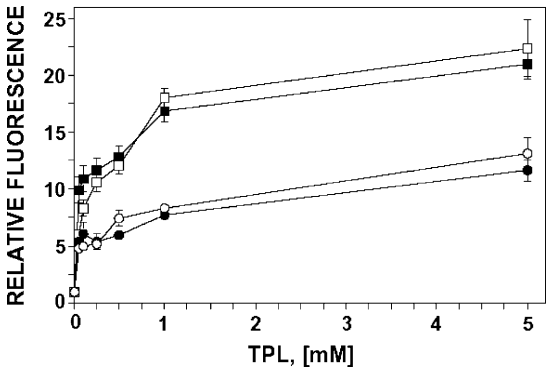

Fig. 3. TPL-induced ROS production. VEC (circle symbols) and VSMC (square symbols), which were preincubated with 5.0 (open symbols) or 23.0 mM glucose (black symbols), were treated with increasing concentrations of TPL for 12 h. The cells were then loaded with H_2DCF and ROS-dependent fluoresnce was determined as described in Section 2. One unit was taken as cell fluorescence in the absence of TPL. Mean ± S.E.M., $n = 3$.

normo- or hyperglycemic condition. Yet, AG (1.0 mM), NAC (5.0 mM), trolox (0.5 mM) and Vitamin C (1.0 mM) reduced significantly the level of protein carbonylation in VEC and VSMC exposed to 33.0 mM glucose (Fig. 2B).

To address the possibility that TPL-induced protein carbonylation resulted from its pro-oxidant interactions we determined the fluorescence generated in its presence with the ROS-sensitive probe H_2DCF. VEC and VSMC, which were maintained at 5.5 or 23.0 mM glucose, were further incubated for 12 h in the absence or presence of increasing concentrations of TPL. The cells were then washed and treated with H_2DCF as described in Section 2. Fig. 3 shows that TPL increased cell fluorescence 13.1 ± 1.4-fold and 22.3 ± 2.6-fold in VEC and VSMC, which were exposed to 5.0 mM glucose, respectively. Under hyperglycemic conditions the respective values were 11.6 ± 0.9-fold and 21.0 ± 1.1-fold. Half maximal fluorescence inducing capacity of TPL was observed at

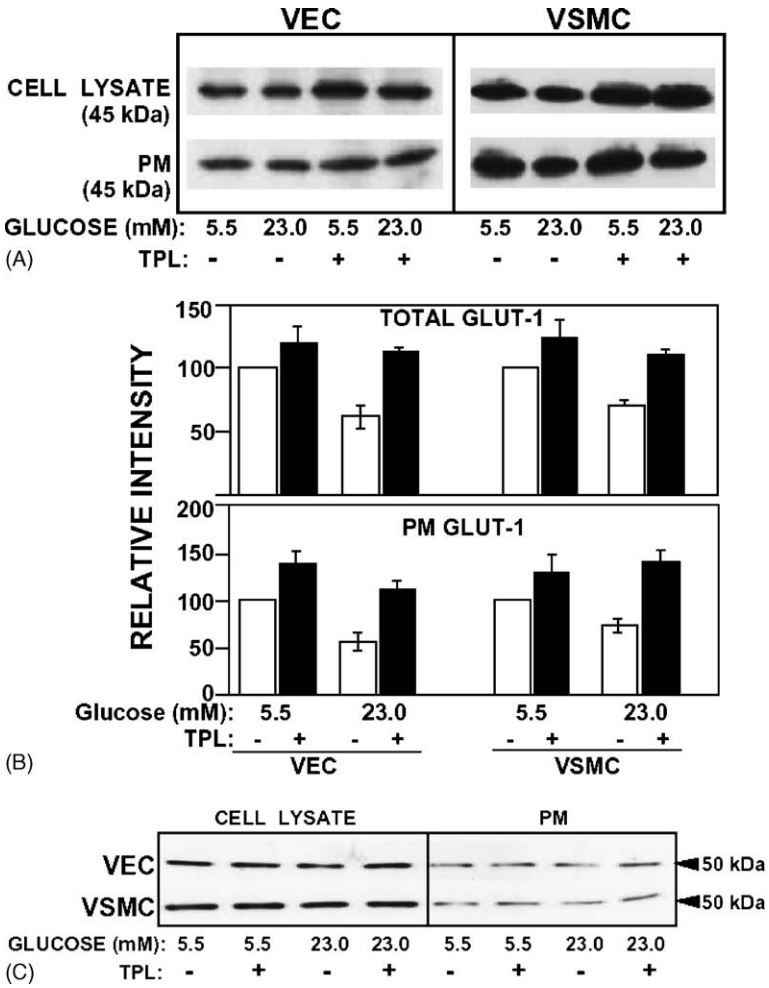

Fig. 4. Effect of TPL on total and plasma membrane-localized GLUT-1. VEC and VSMC were treated with 5.0 mM TPL for 10 h, as described in the legend to Fig. 1. The preparation of total cell lysate and the isolation of cell-surface biotinylated proteins were performed as described previously [38,59]. (A) A representative Western blot of total cell lysate and plasma membrane-associated (PM) GLUT-1. (B) Relative intensities of GLUT-1 signals in total cell lysates and in plasma membranes in the absence (open bars) or presence of 5.0 mM TPL (black bars). The 100% values are assigned to the appropriate cell lysate and PM band intensities from control cells that were not treated with TPL. Means ± S.E.M., $n = 3$. (C) A representative Western blot of total and plasma membrane-localized GLUT-4 in control and TPL-treated VEC and VSMC.

0.25–0.5 mM (Fig. 3). These data indicate that TPL generated ROS and was a potent pro-oxidant in vascular cells under both normo- and hyperglycemic conditions.

3.3. TPL increases GLUT-1 content and plasma membrane localization in VEC and VSMC

TPL-dependent stimulation glucose transport and metabolism may result from an increased expression and/or plasma membrane localization of GLUT-1 in the cells. Thus, GLUT-1 content in whole cell lysates and in the plasma membrane was determined (Fig. 4A and B). As shown before [38], the total cell content and plasma membrane-localized GLUT-1 were reduced in vascular cells exposed to 23.0 mM glucose in comparison to cells at 5.5 mM (VEC: $49 \pm 12\%$ and $44 \pm 15\%$ reduction, respectively $P < 0.05$; VSMC: $31 \pm 5\%$ and $28 \pm 5\%$, respectively, $P < 0.05$). TPL increased the total cell content of the transporter 1.84 ± 0.06-fold and 1.57 ± 0.04-fold under hyperglycemic conditions in VEC and VSMC, respectively ($P < 0.05$, compared to each respective control), while its effects at 5.5 mM glucose were smaller (1.20 ± 0.10-fold and 1.24 ± 0.12-fold for VEC and VSMC, respectively). Concomitantly, TPL increased the plasma membrane abundance of GLUT-1, 2.04 ± 0.09-fold ($P < 0.05$) and 1.90 ± 0.07-fold ($P < 0.05$) under the hyperglycemic conditions in VEC and VSMC, respectively, and 1.36 ± 0.09-fold and 1.28 ± 0.12-fold, respectively, under normoglycemic conditions.

VEC and VSMC also express low levels of the insulin sensitive glucose transporter GLUT-4, whose expression is not altered under hyperglycemic conditions [38]. Western blot analysis of GLUT-4 of total cell lysates and biotinylated cell surface proteins prepared from VEC and VSMC indicated that neither hyperglycemic conditions nor TPL changed the content of GLUT-4 or its plasma membrane abundance (Fig. 4C).

3.4. TPL elevates GLUT-1 mRNA content in VEC and VSMC

A competitive PCR method for GLUT-1 mRNA was used to determine whether the increased expression of GLUT-1 protein in TPL-treated VEC and VSMC under hyperglycemic conditions resulted from an overexpression of GLUT-1 mRNA [38]. Fig. 5 shows the effects of TPL and esculetin (an inhibitor of lipoxygenases) on the relative expression of GLUT-1 mRNA in VEC and VSMC that were preincubated at 23.0 mM glucose for 48 h and exposed to esculetin (ESC: 100 μM) and/or TPL (5.0 mM) during the last 8 h of incubation. As shown before [38], esculetin increased the level of the message 1.5- and 3.8-fold in VEC and VSMC, respectively. The effect of TPL was similar: 1.7- and 3.7-fold increase, respectively. The combined effect of both compounds was additive and increased 3.1- and 7.3-fold in the expression of GLUT-1 mRNA in VEC and VSMC, respectively. These data show that these two agents increase GLUT-1 mRNA expression in distinct mechanisms.

3.5. Effects of TPL on mitochondrial ATP production in VEC and VSMC

Mitochondrial ATP production from glutamic acid and malic acid, as substrates for Complex I in the respiratory chain, and the rate of [^{3}H]dGlc uptake in control and TPL-treated VEC and VSMC were determined. Analysis of the data in Fig. 6 shows $30 \pm 2\%$ and $32 \pm 2\%$ reduction in ATP production in TPL-treated VEC and VSMC that were maintained at 5.5 mM glucose (mean ± S.E.M., $n = 3$; $P < 0.05$, compared to each respective control). This reduction was accompanied with a $31 \pm 2\%$ and $57 \pm 7\%$ increase in the rate of dGlc uptake, respectively (mean ± S.E.M., $n = 3$, $P < 0.05$). TPL reduced mitochondrial ATP production in cells exposed to high glucose

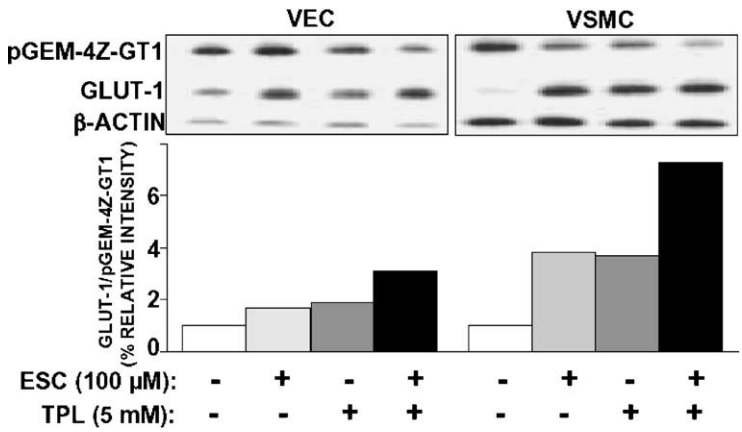

Fig. 5. Effects of TPL and esculetin on GLUT-1 mRNA in VEC and VSMC. Confluent VEC and VSMC cultures were maintained at 23.0 mM glucose for 48 h without or with esculetin (100 μM) and/or TPL (5.0 mM) during the last 8 h of incubation. RNA extraction, cDNA synthesis, PCR of GLUT-1 cDNA in the presence of the competing pGEM-4Z-GT1 plasmid and product analysis are described in Section 2 and by Alpert et al. [38]. PCR product image and relative intensities of GLUT-1 PCR product vs. the competing pGEM-4Z-GT1 PCR product are shown. The ratio of GLUT-1 to pGEM-4Z-GT1 at 23.0 mM glucose for each type of cell was taken as 1 unit.

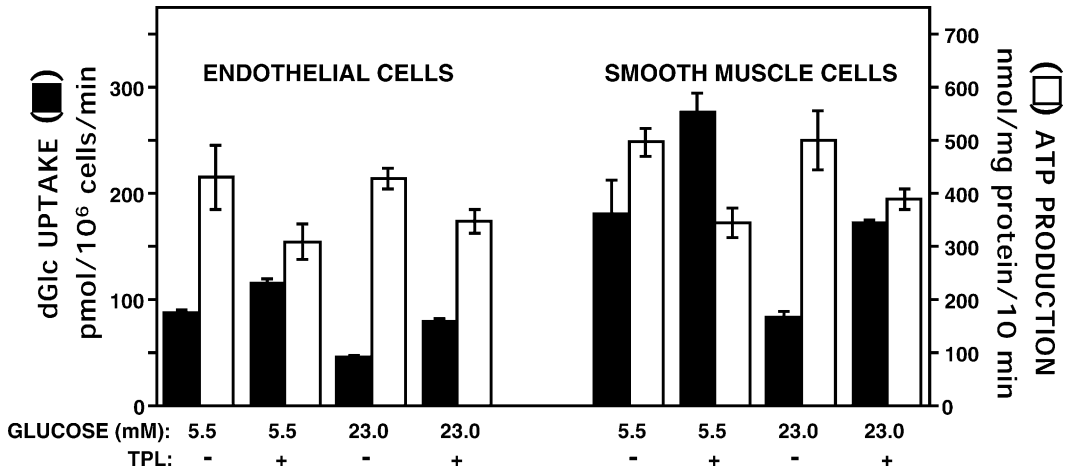

Fig. 6. Effect of TPL on the rate of mitochondrial ATP production and hexose uptake in VEC and VSMC. Following a treatment of VEC and VSMC, as described in the legend to Fig. 1, the cells were taken for the ATP production and [^{3}H]dGlc uptake assays as described in Section 2. Open columns, ATP production (nmol/10^6 cells/10 min); black columns, [^{3}H]dGlc uptake (pmol/10^6 cells/min). Means ± S.E.M., $n = 3$.

by 21 ± 2% and 24 ± 3% ($P < 0.05$) for VEC and VSMC, respectively. In parallel, TPL increased the rate of hexose transport by 65 ± 3% and 106 ± 5% ($P < 0.05$) in VEC and VSMC, respectively.

The level of ATP production from endogenous cell substrates in permeabilized VEC and VSMC in the absence of glutamic acid and malic acid was low and accounted for less than 5% of the total amount of ATP generated from this couple substrates. Other control experiments were performed in the presence of rotenone (5 μM), an inhibitor of Complex I in the respiratory chain, or cyanide (KCN, 0.3 mM), an inhibitor of Complex IV. Both reduced (80 and 95%, respectively) the generation of ATP from glutamic acid and malic acid.

To further investigate the relationship between reduced mitochondrial ATP production and augmented rate of glucose transport the cells were treated with the uncoupler dinitrophenol (DNP), which prevents the formation of proton gradients across the mitochondrial membrane, and was shown to increase the rate of glucose transport in cells [45]. VEC and VSMC maintained at 23.0 mM glucose for 48 h that were exposed to 0.1 mM DNP during the last 8 h of incubation reduced the rate of mitochondrial ATP production by 50–75%. In parallel, the rate of dGlc uptake was augmented 1.56 ± 0.12-fold and 1.61 ± 0.13-fold (mean ± S.E.M., $n = 3$) for VEC and VSMC, respectively. These data are consistent with previous reports in which impairments of mitochondrial function augmented the rate of glucose transport as a compensatory mechanism [39].

3.6. Lack of effect of TPL on 12- and 15-LO activity

TPL, being an iron-ion chelator, could also increase the rate of glucose transport in vascular cells by inhibiting the enzyme 12-LO and the intracellular level of its product 12-HETE. Thus, HPLC analysis of 12-HETE was performed on culture medium extracts from VEC and VSMC cultures that were preexposed to 23.0 mM glucose for 48 h and to 5.5 mM TPL during the last 10 h of incubation. The level of secreted 12-HETE during the 10 h incubation period was not altered significantly in the presence of TPL (Table 2), indicating that the enzymatic activity of 12-LO remained unaltered.

Table 2
Lack of effect of TPL on levels of secreted 12-HETE

Cell type	Treatment	12-HETE
VEC	Control	12.6 ± 3.4
	Control + TPL	10.7 ± 3.6^{ns}
VSMC	Control	48.3 ± 13.6
	Control + TPL	57.9 ± 4.0^{ns}

VEC and VSMC were maintained at 23.0 mM glucose and treated with 5.5 mM TPL as described in the legend to Fig. 1. At the end of the incubation period culture media were collected, extracted and taken for HPLC analysis, as described in Section 2. The data is given as ng of 12-HETE/10^6 cells. ns: not significantly different ($P > 0.05$) from the respective control value. Mean ± S.E.M., $n = 3$.

4. Discussion

Diabetes-induced vascular cell dysfunction has been linked to an excessive production of ROS and uncoupling of eNOS, which leads to an overproduction of superoxide anions rather than NO radicals, and subsequently blunted acetylcholine-induced vasodilatation [17,23]. TPL-dependent superoxide dismutation or scavenging activity may provide protection against such effects: It ameliorates endothelial cell dysfunction in renal afferent arterioles [4], aortae [2] and coronary arteries [28] of diabetic rats. TPL also prevented hypertension in angiotensin-treated rats [29], in spontaneously hypertensive rats [30] and in nephrectomized rats [32]. In addition, it also protected

against oxidative damage in beating cardiomyocytes in culture [46] and reduced the infarct size in rodent models of regional myocardial ischemia and reperfusion [47]. Therefore, hydrophobic nitroxide derivatives are being developed and assessed as potential agents to prevent vascular cell dysfunction in diabetes and other oxidative stress-induced pathologies [48]. However, potential pro-oxidant adverse interactions of TPL, especially in the mitochondrion, which alter ATP production and affect the glucose transport system and energy metabolism in vascular cells, have not been addressed.

This study describes a TPL-dependent stimulation of glucose transport, an augmented expression of GLUT-1 protein and mRNA and an increased abundance of the transporter in the plasma membrane of VEC and VSMC under both normo- and hyperglycemic conditions. In parallel, TPL reduced the rate of mitochondrial ATP production, but did not alter the enzymatic activity of 12-LO. Unlike the other antioxidants, TPL failed to reduce protein carbonylation in vascular cells exposed to high glucose levels and, in fact, increased it significantly, while generating ROS. Due to the lack of effect of Vitamin C, trolox, *N*-acetyl cysteine and aminoguanidine on glucose transport it is concluded that the glucose transport system in the vascular cells is not sensitive to ROS generated under hyperglycemic conditions. Moreover, TPL-induced glucose transport augmenting activity results from its potent pro-oxidant rather than antioxidant interactions.

Indeed, in contrast to reports on favorable antioxidative effects of TPL there are other studies on adverse cellular effects of TPL: Gariboldi et al. reported that TPL inhibited growth of neoplastic cells by inducing an irreversible damage [49]. When added to a human leukemia cell line (HL60) or Yoshida sarcoma cells it inhibited dose- and time-dependently cell growth and induced apoptosis [49,50]. Monti et al. observed TPL accumulation in mitochondria of HL60 cells in a direct correlation to its concentration in the culture medium and an impairment of mitochondrial oxidative-phosphorylation by (a) inhibiting Complex I, and to a lesser extent Complexes II and IV, in the respiratory chain and causing reduction in ATP production, (b) decreasing mitochondrial membrane potential, and (c) reducing both cellular and mitochondrial glutathione pools [51]. Noteworthy is Floyd's early observation that nitroxides interact with the electron transport carriers in the mitochondrion [52]. These studies and the present results confirm the fact that TPL can generate free radical, impair mitochondrial functions, and alter essential cell functions [37,49]. Several interactions of TPL may explain its pro-oxidative activity: The net TPL scavenging reaction, which involve its transition between a radical and an ammonium cation forms hydrogen peroxide [37]. Inhibition of Complex I in the mitochondrion has been shown to contribute to ROS formation [51]. TPL has also been implicated in depleting both total cellular and mitochondrial glutathione (GSH) pools in cells. Such depletion may increase mitochondrial ROS, which are generated during the process of aerobic respiration [51]. It remains to be investigated whether TPL induces ROS production also by activating other critical mitochondrial and cytoplasmic reactions, such as of NADPH oxidase.

Our findings on TPL-induced relative ATP depletion explain the induction in GLUT-1 expression. This transporter is classified as a stress-protein whose expression is increased in response to a variety of stressful stimuli [53] including hypoxia and inhibition of mitochondrial respiratory chain-linked oxidative phosphorylation, at any of its steps [39,54]. This induction compensates for the reduced capacity of cells to generate adequate ATP supply in mitochondria by increasing glucose flux and ATP synthesis through the glycolytic pathway. This study shows a similar compensatory mechanism that is operated in TPL-treated vascular cells.

The smaller inhibitory effect of TPL on mitochondrial ATP production under hyperglycemic conditions suggests that the respiratory chain complexes may be partially protected, perhaps by limiting the accumulation of TPL and/or its replenishment via the hydroxylamine-nitroxide cycle in the mitochondrion [55]. Also, the binding of hexokinases to a pore protein in the outer and inner mitochondrial membranes augments glucose-phosphorylating kinetics of this enzyme [56]. Defects in the respiratory chain and disruption of the mitochondrial membrane potential result in a decreased interaction of hexokinases with the mitochondrial docking protein and reduced the enzyme activity [57]. The finding reported by Monti et al. on a TPL-induced reduction of mitochondrial membrane potential [51] and the present results on mitochondrial dysfunction in VEC and VSMC under a similar treatment suggest that the hexokinase reaction in TPL-treated cells might also be impaired. This can aggravate the limiting energetic conditions imposed upon the cells and contribute further to the overexpression of GLUT-1. This particular mechanism remains to be investigated.

We have shown previously that inhibition of the arachidonic metabolizing enzyme 12-LO and reduced production of its metabolite 12-HETE operate a cellular mechanism that increases the total cell content of GLUT-1 protein and mRNA and its plasma membrane localization, and consequently upregulate the glucose transport system in VEC and VSMC [38]. Because iron chelators can inhibit LO, the possibility that TPL inhibited lipoxygenases by oxidizing the iron ion in their catalytic centers was also addressed [58]. TPL did not alter 12-HETE production, indicating that an inhibition of 12-LO was not linked to the effects of TPL in vascular cells. Moreover, the combined effects of lipoxygenase inhibition (by esculetin) and TPL on GLUT-1 mRNA expression and glucose transport were additive, indicating that each agent activates a distinct cellular mechanism to enhance the expression and/or stability of GLUT-1 mRNA.

It is important to note that the concentrations and doses of the water-soluble TPL used in the present study and other cited above are higher than what is usually considered a therapeutic level. Nevertheless, the doses required for achieving minimal effective concentrations both in in vitro and in vivo model systems may be reduced considerably with the ongoing development of lipid-soluble nitroxide derivatives.

In summary, TPL-dependent upregulation of glucose transport and metabolism in VEV and VSMC is related to a reduced rate of mitochondrial ATP synthesis. These effects of TPL were present both under normo- and hyperglycemic conditions and may result from pro-oxidative interactions of TPL, which significantly increase free radical formation and protein carbonylation. These findings on the damaging effects of TPL also reflect Glebska's et al. [26] advice for a careful evaluation of deleterious cellular effects of nitroxides, which are related to their potential pro-oxidant properties. Therefore, this study calls for an assessment of the view that TPL functions mainly as an antioxidant in vascular cells and for an evaluation of its pro-oxidant interactions, which may alter essential cell interactions, such as mitochondrial functions, glucose transport and energy metabolism.

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