

ORIGINAL INVESTIGATION

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Effects of some anti-diabetic and cardioprotective agents on proliferation and apoptosis of human coronary artery endothelial cells

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Abstract

Background: The leading cause of death for patients suffering from diabetes is macrovascular disease. Endothelial dysfunction is often observed in type 2 diabetic patients and it is considered to be an important early event in the pathogenesis of atherogenesis and cardiovascular disease. Many drugs are clinically applied to treat diabetic patients. However, little is known whether these agents directly interfere with endothelial cell proliferation and apoptosis. This study therefore aimed to investigate how anti-diabetic and cardioprotective agents affect human coronary artery endothelial cells (HCAECs).

Methods: The effect of anti-diabetic and cardioprotective agents on HCAEC viability, proliferation and apoptosis was studied. Viability was assessed using Trypan blue exclusion; proliferation in 5 mM and 11 mM of glucose was analyzed using [³H]thymidine incorporation. Lipoapoptosis of the cells was investigated by determining caspase-3 activity and the subsequent DNA fragmentation after incubation with the free fatty acid palmitate, mimicking diabetic lipotoxicity.

Results: Our data show that insulin, metformin, BLX-1002, and rosuvastatin improved HCAEC viability and they could also significantly increase cell proliferation in low glucose. The proliferative effect of insulin and BLX-1002 was also evident at 11 mM of glucose. In addition, insulin, metformin, BLX-1002, pioglitazone, and candesartan significantly decreased the caspase-3 activity and the subsequent DNA fragmentation evoked by palmitate, suggesting a protective effect of the drugs against lipoapoptosis.

Conclusion: Our results suggest that the anti-diabetic and cardioprotective agents mentioned above have direct and beneficial effects on endothelial cell viability, regeneration and apoptosis. This may add yet another valuable property to their therapeutic effect, increasing their clinical utility in type 2 diabetic patients in whom endothelial dysfunction is a prominent feature that adversely affect their survival.

Keywords: Diabetes, Endothelium, Coronary artery disease, Insulin, Statin, Metformin, Lipids, AT₁-receptor antagonist, Peroxisome proliferator-activated receptor gamma agonist

Background

The prevalence of diabetes among adults worldwide was estimated in 2010 to 6.4%, thus affecting 285 million adults. This figure is predicted to rise to 7.7%, in numbers 439 million, by 2030 [1]. In patients with diabetes, the major cause of death is macrovascular disease [2,3], and in individuals with type 2 diabetes, the main etiology for up to 75% of the mortality is atherosclerotic

cardiovascular disease [4]. In contrast to microangiopathies (e.g. nephropathy and retinopathy), where the causal relation to hyperglycemia is well supported, the link between hyperglycemia and macroangiopathy is uncertain, at least in terms of the possibility of reducing macrovascular morbidity solely by reducing hyperglycemia. Most patients with diabetes are consequently being treated with one or more antidiabetic drugs, a lipid-lowering statin and an ACE inhibitor or angiotensin receptor antagonist for hypertension and/or albuminuria.

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The endothelium is composed of a monolayer of cells that line the lumen of blood vessels and form a physical barrier between circulating blood and the vascular smooth muscle cells. The endothelium plays a very important role in maintenance of vascular integrity by protecting the vessels from activation of clotting and proinflammatory factors. It also participates in the regulation of blood flow and blood pressure [5]. Loss of physiological features of the endothelium, such as its preference to support vasodilatation, fibrinolysis and antiaggregation, is referred to as endothelial dysfunction [6], which has been observed in diabetes (type 1 and type 2), in obesity and in patients with insulin resistance. In fact, the extent of endothelium-dependent vasodilatation correlates in obese and insulin resistant subjects with their individual insulin sensitivity [7,8]. Endothelial dysfunction has thus emerged as an important early target for preventing atherosclerosis and cardiovascular disease [9].

Hyperglycemia and hyperlipidemia are important factors in the development of endothelial dysfunction. Free fatty acids (FFAs), formed during lipolysis from triglycerides, and hyperglycemia are known to impair the endothelial-dependent vasodilatation [10,11]. Increased plasma levels of FFAs and glucose are characteristic features in patients with type-2 diabetes. Both factors are known induce apoptosis of endothelial cells and endothelial cell death is believed to be involved in, and contribute to, endothelial dysfunction and atherosclerosis [12-14].

Current anti-diabetes drugs are mainly aimed to correct hyperglycemia by promoting pancreatic β -cell insulin secretion, increasing insulin sensitivity, or reducing intestinal glucose uptake and hepatic gluconeogenesis. Apart from insulin, glimepiride, a third generation sulfonylurea [15], pioglitazone, a peroxisome proliferator-activated receptor gamma (PPAR- γ) agonist, and a member of the thiazolidinedione family (TZD) [16], and metformin, a biguanide, are used for treatment of type 2 diabetes [9]. Candesartan, an angiotensin II receptor antagonist, and rosuvastatin, a competitive inhibitor of the enzyme HMG-CoA reductase, are used for management of hypertension and hyperlipidemia, respectively [17,18]. In addition, we have also studied BLX-1002, a novel thiazolidinedione with no structural resemblance to other TZDs [19], which does not appear to affect PPARs. There is evidence that BLX-1002 can improve hyperglycemia in diabetic animal models without the body weight gain typically associated with PPAR γ -mediated adipocyte differentiation [19,20]. Not much is known about BLX-1002, but it has been shown to potentiate insulin secretion from islets in a phosphatidylinositol 3-kinase (PI3K)-dependent manner. BLX-1002 also activates AMP-activated protein kinase (AMPK) [20] perhaps through its ability to inhibit the mitochondrial complex 1 [19,21].

Compared to studies on the actions of the above drugs on glycemia, little has been done regarding their direct actions on proliferation and apoptosis of human coronary artery endothelial cells (HCAECs). Understanding the effects of these agents is important as endothelial growth and apoptosis are involved in endothelial repair and function. Disruption of the intimal layer subjects the arterial wall to greater risk for macrovascular disease [14], the most common etiology for morbidity and mortality in diabetic patients [15]. Therefore, the aim of our study was to investigate the effects of drugs used in treatment of diabetic patients on proliferation and lipotoxicity-induced apoptosis in HCAECs.

Methods

Materials

Clonetics™ HCAECs, culture medium EGM-2 MV and cell culture supplements were purchased from Lonza (Basel, Switzerland). Insulin lispro was from Eli Lilly and Company (Indianapolis, IN), C-peptide was generously provided by Prof. John Wahren (Creative Peptides, Inc., Stockholm, Sweden), and pioglitazone was graciously donated by Takeda Pharmaceuticals North America, Inc. (Lincolnshire, IL). Metformin, glimepiride, sodium palmitate, and bovine serum albumin (BSA) (fatty acid free) were purchased from Sigma-Aldrich (St. Louis, MO). Rosuvastatin and candesartan were kindly given by Astra-Zeneca (London, United Kingdom). BLX-1002 was graciously donated by Bexel Pharmaceuticals, Inc. (Union City, CA). [³H]thymidine was bought from Amersham Biosciences (Piscataway, NJ), EnzChek® caspase-3 activity assay kits from Molecular Probes®, Life Technologies (Carlsbad, CA), DNA fragmentation ELISA kits from Roche Diagnostics (Mannheim, Germany), and DC™ Protein Assay from BioRad Laboratories (Hercules, CA).

Cell culture

HCAECs, isolated from normal human coronary arteries [22], were grown in EGM-2 MV medium supplemented with hydrocortisone, human epidermal growth factor (hEGF), 5% fetal bovine serum (FBS), vascular endothelial growth factor (VEGF), human fibroblast growth factor (hFGF)-B, R3-insulin-like growth factor (IGF)-1, ascorbic acid and gentamicin/amphotericin-B at 37°C in a humidified (5% CO₂, 95% air) atmosphere as recommended by the supplier. Passage 5-12 were used in the study for evaluation of cell viability, [³H]thymidine incorporation rates, DNA fragmentation ELISA and caspase-3 activity assays. Confluent cultures were detached by trypsinization and seeded onto tissue culture dishes and grown until 80-90% confluence. Cells were incubated overnight in serum-deficient EGM medium containing 0.5% FBS and 2 mM L-glutamine prior to 24 or 48 h incubation in the

presence or absence of the desired agents. The use of the cells was approved by the Research Ethics Committee, Stockholm South, Dnr: 232/03.

Cell viability

Cells were incubated with serum deficient medium, in the presence or absence of the drugs for 48 h. Cell number was manually counted in a hemocytometer and cell viability assessed by Trypan blue exclusion.

[³H]thymidine incorporation

Rates of [³H]thymidine incorporation into DNA were analyzed as previously described [22,23] and used as a measure of DNA synthesis. In brief, cells were cultured until 80% confluence. After serum starvation over night, cells were incubated in the presence of the agents or vehicle for 24 h at 5 mM or 11 mM glucose to simulate a normoglycemic and hyperglycemic milieu, respectively. Cells were pulsed with [³H]thymidine (1 μCi/ml) 8 h prior to the end of the incubation. Cells were then collected and homogenized through ultrasonication. The labeled cells were precipitated in ice-cold 10% trichloroacetic acid (TCA). The precipitate was washed with 10% TCA and [³H]thymidine incorporation into DNA was measured using a microplate scintillation and luminescence counter (Wallac MicroBeta[®] Trilux, PerkinElmer) [24]. The protein concentration of the samples was measured using DC[™] Protein Assay (Bio-Rad Laboratories).

Caspase-3 activity

Cells were cultured to 90% confluence. After incubation in serum deficient medium overnight, cells were pre-treated for 1 hour with the drugs or solvents, after which the incubation was continued for 24 h in the presence of 0.125 mM palmitate/0.25% BSA or vehicle [13]. Caspase-3 activity, a measure of apoptosis, was evaluated using the EnzChek[®] Caspase-3 Assay Kit (Molecular Probes[®], Life Technologies) according the manufacturer's instructions. The assay is based on the 7-amino-4-methylcoumarin-derived substrate Z-DEVD-AMC, which yields a fluorescent product (excitation/emission ~342/441 nm) upon proteolytic cleavage by active caspase-3. All results were normalized to the protein concentration of the corresponding sample using DC[™] Protein Assay (Bio-Rad Laboratories).

DNA fragmentation

Cells were cultured to 90% confluence and treated in the same way as described for the caspase-3 activity assay. Cell apoptosis was analyzed using the Cell Death Detection Kit plus (Roche Diagnostics) according the manufacturer's instructions. The Cell Death Detection Kit measures cytoplasmic DNA-histone nucleosome complexes generated during apoptotic DNA fragmentation. Samples were

measured at 405 nm and corrected for background signals caused by irregular microtiter plates or light scattering due to solid particles in the solution at the reference wavelength of 492 nm. Separate wells were seeded for protein concentration measurements using DC[™] Protein Assay (Bio-Rad Laboratories). Absorbance data was normalized to the protein concentration of the corresponding treatment.

Statistical analysis

Results are expressed as mean ± SEM. Statistical analysis was performed using Student's *t*-test or ANOVA, as appropriate. *P* < 0.05 was considered statistically significant.

Results

Agents that were used in this study were the following: insulin lispro (100 pM, 1 nM), C-peptide (1 nM), pioglitazone (2.5 μM), metformin (500 μM), glimepiride (1 μM), rosuvastatin (10 nM), candesartan (100 nM) and BLX-1002 (1 μM). Of the compounds tested, we were unable to detect any effect of C-peptide on either proliferation or apoptosis of the cells. All agents have been tested in all conditions, but only compounds that were able to exert significant effects are displayed in the graphs.

Cell viability of HCAECs is increased by the agents

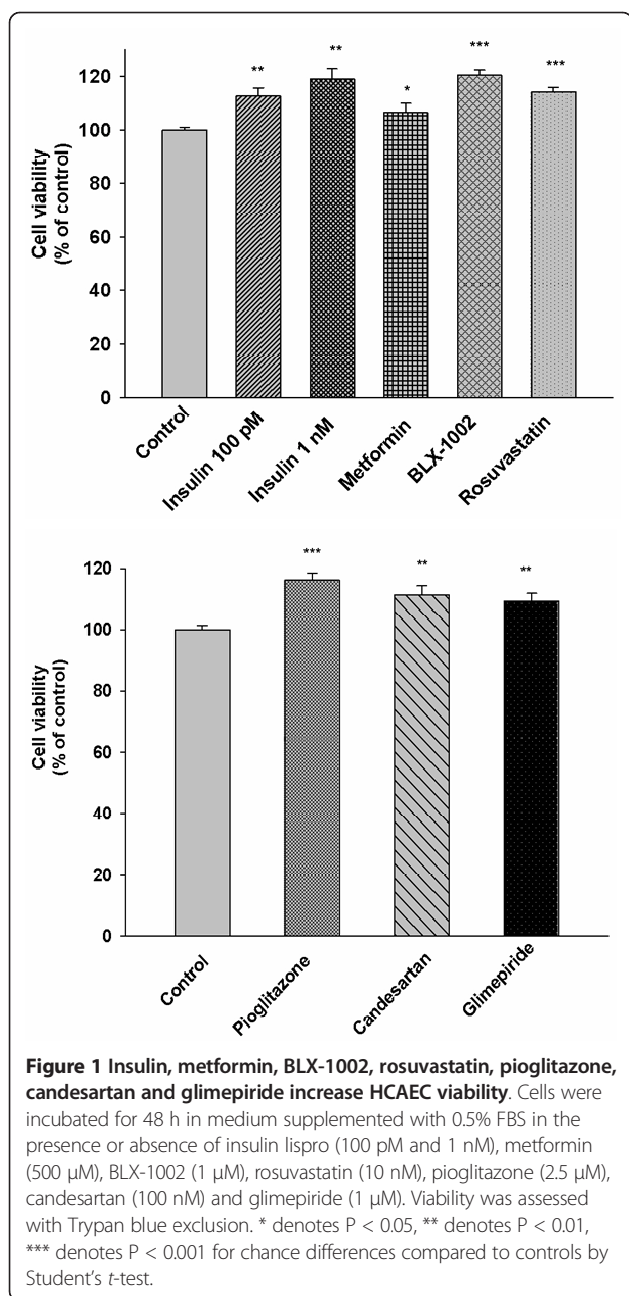
As shown in Figure 1, among the agents tested, BLX-1002 and insulin (the latter at both 100 pM and 1 nM) increased cell viability. Exposure of the cells to metformin also resulted in a slight, but statistically significant, increase in cell viability. Under the same conditions, cell viability was also augmented by rosuvastatin, pioglitazone, candesartan and glimepiride.

Insulin, BLX-1002, metformin and rosuvastatin stimulate proliferation of HCAECs at normal glucose concentration

Since the increased viability noted above (Figure 1) could be explained by increased proliferation, decreased apoptosis, or a combination thereof, we investigated if the drugs exert any effect on HCAECs' DNA synthesis. Rates of [³H] thymidine incorporation were analyzed after a 24 hour exposure to the agents at 5 mM glucose. The mitogenic effect of the compounds was also verified by measuring protein concentrations of the samples to reflect an increase in cell number. As shown in Figure 2, insulin, BLX-1002, metformin and rosuvastatin increased both DNA synthesis and protein concentration significantly.

Insulin and BLX-1002 stimulate proliferation of HCAECs at high glucose

Since insulin, BLX-1002, metformin and rosuvastatin were able to increase the proliferation of HCAECs in 5 mM glucose (Figure 2), we further investigated whether they had any effect on cell proliferation at high glucose. HCAECs were therefore exposed to 11 mM of glucose,



to simulate a diabetic setting, in the presence or absence of the agents. Similar to the effect observed at 5 mM glucose, insulin and BLX-1002 were both able to increase DNA synthesis and protein concentration (Figure 3). In contrast to their effects at 5 mM glucose, metformin and rosuvastatin did not exert any effect on cell proliferation under these conditions (data not shown).

Insulin, BLX-1002, metformin, pioglitazone and candesartan suppress lipoapoptosis in HCAECs

Plasma FFA levels are increased in type-2 diabetic patients and the FFA palmitate is known to induce

apoptosis of endothelial cells [13]. We therefore evaluated how the agents affected the activity of cleaved caspase-3, a crucial mediator of apoptosis [25], in HCAECs after 24 hour exposure to 0.125 mM palmitate. As shown in Figure 4, palmitate significantly increased caspase-3 activity, thus confirming its pro-apoptotic effect, in HCAECs. Insulin, BLX-1002, metformin, pioglitazone and candesartan significantly decreased the palmitate-induced caspase-3 activation, suggesting a protective effect of the drugs against lipotoxicity in HCAECs. Glimepiride significantly decreased basal caspase-3 activity with ~20% (data not shown), which might explain why an increased viability of the cells in the presence of glimepiride is noted (Figure 1).

To corroborate the anti-apoptotic effect of the agents above, we investigated their influence on palmitate-induced DNA fragmentation by analyzing cytoplasmic DNA-histone nucleosome complexes generated during apoptosis. Palmitate alone gave rise to a markedly increased apoptosis of the HCAECs (Figure 5). Co-incubation with insulin, BLX-1002, metformin, pioglitazone or candesartan significantly countered the palmitate-induced apoptosis, thus confirming the protective effect of these agents against lipoapoptosis in HCAECs.

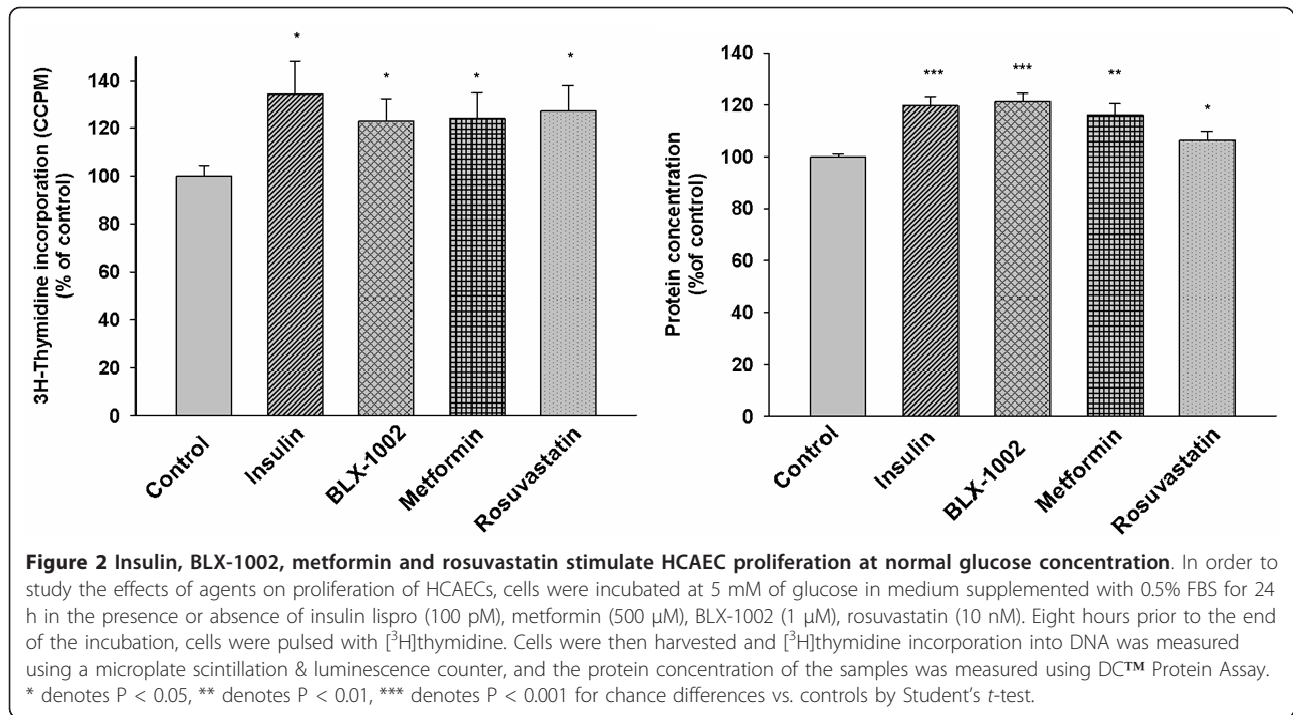
Discussion

Our major findings in this study are that insulin, BLX-1002, metformin and rosuvastatin exert positive effects on regeneration and viability of HCAECs. Insulin, BLX-1002, metformin, pioglitazone and candesartan also conferred protection of HCAECs against lipotoxicity.

Proliferation of HCAECs is induced in normal and high glucose levels

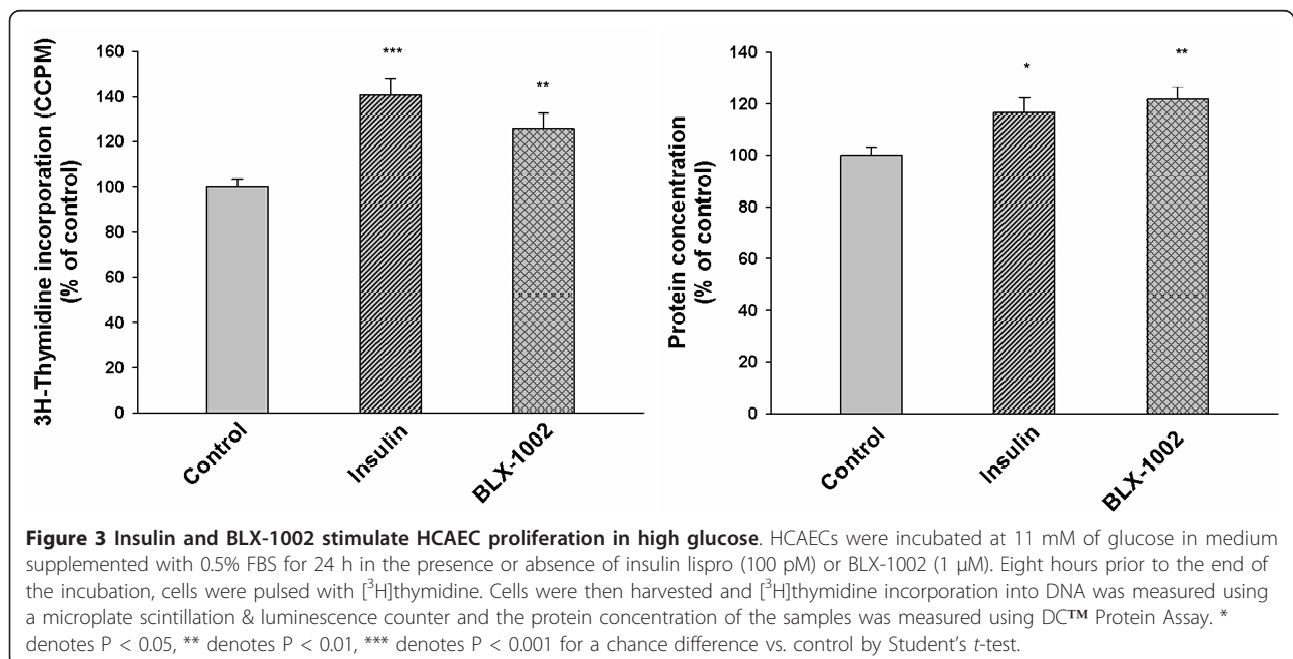
By studying how the agents affect DNA synthesis, through analysis of the incorporation of radiolabeled thymidine into DNA, we found that insulin, BLX-1002, metformin and rosuvastatin increased the HCAECs' DNA synthesis and subsequently the protein concentration in 5 mM glucose. Taken together, this suggests that the above agents stimulate HCAEC proliferation at normal glucose concentrations. The mitogenic effect seen with the compounds probably explain, at least in part, the increased viability.

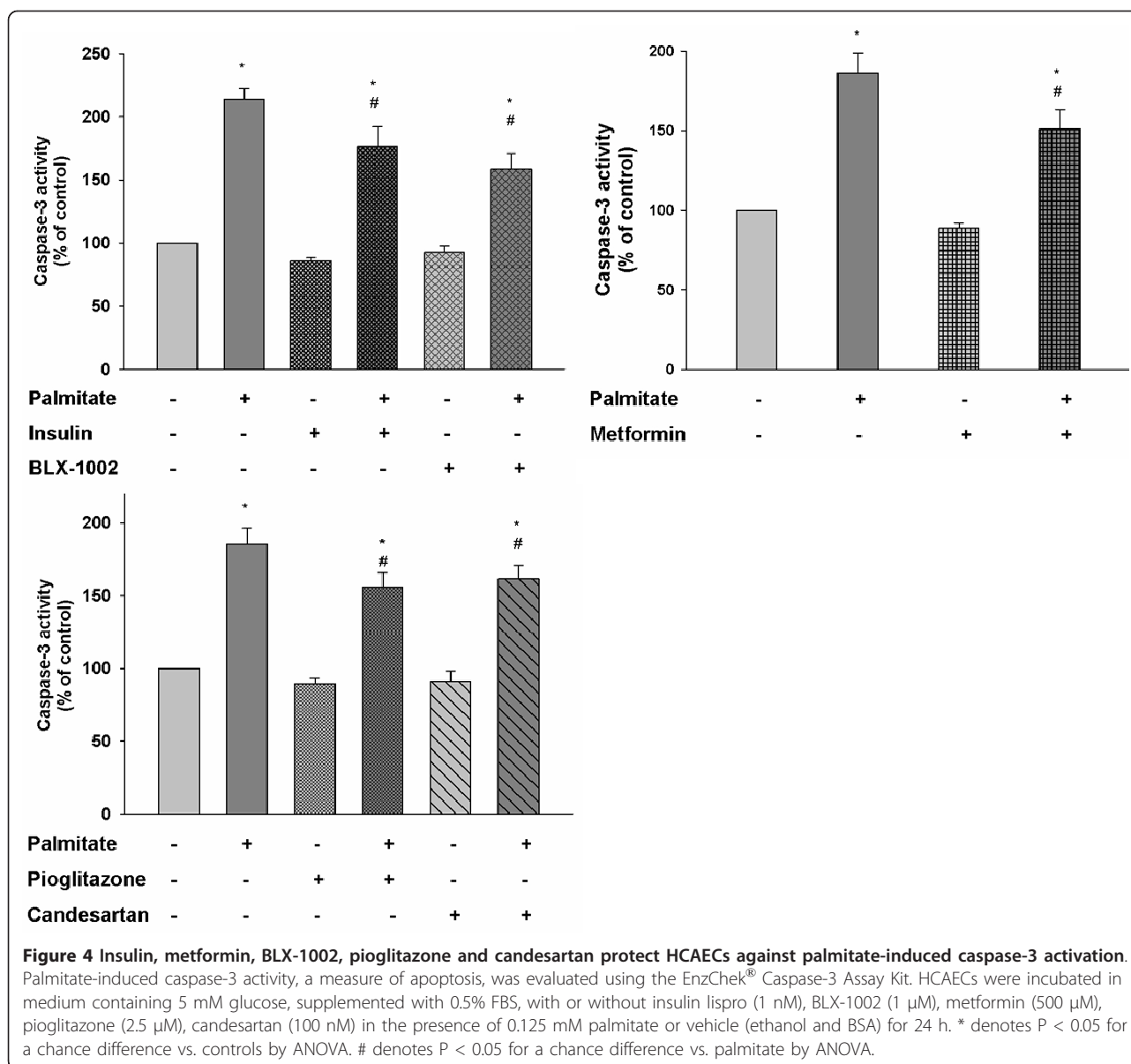
Kageyama *et al.* recently showed that when HCAECs were exposed to glucose concentrations ranging from 5.6-16.7 mM apoptosis was dose dependently increased. This was also coupled to an increased expression of the death receptors TNF-R1 and Fas [26]. High glucose is also known to cause oxidative stress through generation of reactive oxygen species (ROS) in HCAECs as recently shown by Serizawa *et al.* [27]. This might be due to on uncoupling of endothelial nitric oxide synthase (eNOS) and increased activity of the NADPH oxidase [27]. Increased levels of glucose might also cause proliferative



dysfunction of endothelial cells [28,29], which is believed to contribute to premature development of atherosclerosis [30]. We therefore wanted to test if the above agents also stimulate HCAEC proliferation at 11 mM glucose, simulating a diabetic milieu. We found that insulin and BLX-1002 were the only two agents that could promote cell proliferation in high glucose. The mitogenic effect of the agents in HCAECs may prove beneficial in

dampening or delaying coronary atherosclerosis, by rapidly covering a vascular wall lesion with endothelium and thus protecting it from further atherothrombotic events. It may also be favorable for diabetic patients with coronary artery disease undergoing percutaneous coronary revascularization and treated with drug-eluting stents. It is believed that delayed re-endothelization is a major underlying problem for late-stent thrombosis [31] and





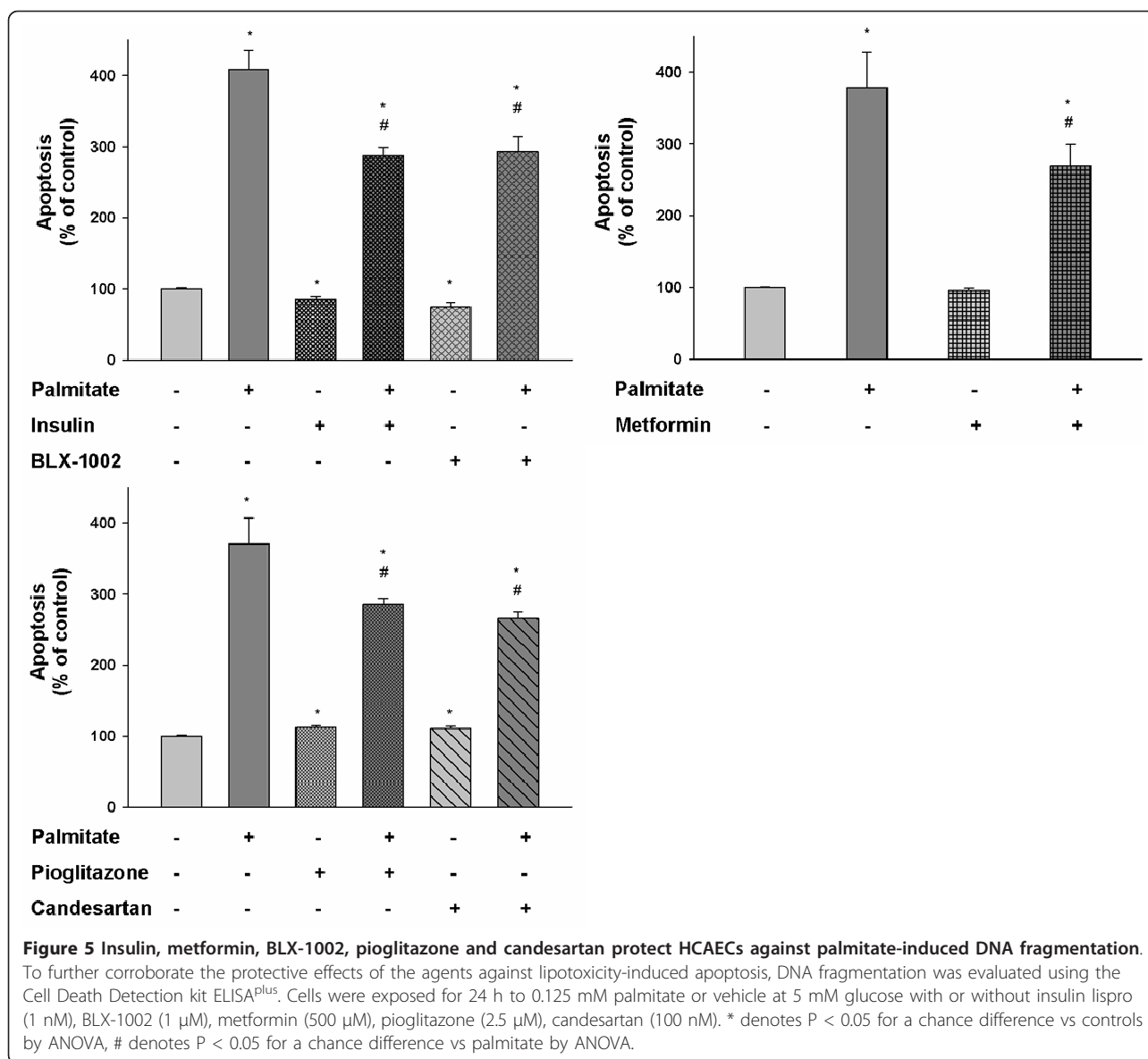
that rapid re-endothelization is essential for preventing restenosis, which is the major limitation with this treatment [32].

The underlying mechanisms involved in the agents' proliferative effects were not investigated in this scanning study, but will be subject to future work. However, the effects are consistent with previous findings insofar that insulin at supraphysiological concentrations increases [³H]thymidine incorporation in endothelial cells [33]. Insulin and rosuvastatin have also been shown to activate the PI3K/Akt pathway, a pathway that is well known to be involved in cell survival and proliferation [18,22,34-36]. BLX-1002 is a novel compound of the TZD family, but with no apparent PPAR affinity. We

have previously demonstrated that it can signal through PI3K and is able to activate AMPK in insulin-secreting cells [20]. Also, metformin is a known activator of AMPK, and AMPK activates eNOS [37], an important factor for proliferation of HCAECs [22].

HCAECs are protected from lipoapoptosis

Plasma levels of FFAs are typically elevated in diabetic patients and FFAs are known to impair endothelial-dependent vasodilatation [11,38]. FFAs are also known to cause apoptosis of endothelial cells [13,38]. Apoptosis of HCAECs might disrupt the endothelial monolayer of the coronary artery wall and might contribute to a proinflammatory environment that could lead to premature



endothelial dysfunction and unfolding of coronary atherosclerosis [13,38]. We therefore addressed whether the above agents protect HCAECs against apoptotic cell death caused by the fatty acid palmitate, simulating diabetic hyperlipidemia. Our results are commensurate with other reports in the literature. Insulin has previously been shown to protect human umbilical vein endothelial cells (HUVECs) from palmitate-induced apoptosis through activation of the PI3K/Akt pathway [38]. BLX-1002, whose functions are essentially unknown, has been shown to promote insulin secretion in pancreatic β-cells, an effect that involves PI3K and AMPK activation [20]. Since both PI3K and AMPK convey anti-apoptotic signals in other cell types [38-41], these pathways may also be operative in HCAECs. This also holds true for

metformin, which can activate AMPK and indeed, specific activation of AMPK has been shown to protect bovine aortic endothelial cells from palmitate-induced apoptosis [41]. The anti-apoptotic effect of pioglitazone observed in the present study is consistent with findings in both HUVECs and endothelial progenitor cells (EPCs) [42,43]. Although the mechanisms behind such effect remain unclear and will be addressed in forthcoming studies, palmitate induces ROS in endothelial cells [41] and pioglitazone has been shown to decrease ROS formation in such cells [44]. This might contribute to its anti-apoptotic effect since oxidative stress is known to induce cell death [14,44]. Pioglitazone has also been shown to protect HCAECs from tumor necrosis factor alpha induced caspase-3 activity and apoptosis [45]. To the best of our

knowledge, there is no literature regarding putative effects of candesartan on lipoapoptosis of endothelial cells. But, in support to our findings, candesartan has been shown to protect from hypoxia-induced endothelial cell apoptosis through pathways involving increased eNOS expression and decreased caspase-3 activity [17]. Candesartan's ability to modulate the caspase-3 activity might also contribute to its positive effects on cell viability.

Conclusions

Abnormal proliferation and apoptosis of endothelial cells are involved in endothelial dysfunction, damage and repair. It thus contributes to the premature development of atherosclerosis and vascular complications in diabetes, making it important to understand the influence of currently applied anti-diabetic and cardioprotective agents on endothelial function. Our results suggest that drugs such as insulin, metformin, rosuvastatin, pioglitazone and candesartan that are oftentimes used in the clinical management of diabetic patients have direct and beneficial effects human coronary artery endothelial cells by promoting their proliferation and conferring protection against lipotoxicity. These findings may add yet another valuable property to their therapeutic effects, increasing their clinical utility in type 2 diabetic patients in whom endothelial dysfunction is a prominent feature that adversely affect their survival.

Abbreviations

AMPK: AMP-activated protein kinase; BSA: Bovine serum albumin; eNOS: Endothelial NOS; EPC: Endothelial progenitor cells; FBS: Fetal bovine serum; FFA: Free fatty acid; HCAEC: Human coronary artery endothelial cells; HUVEC: Human umbilical vein endothelial cells; hEGF: Human epidermal growth factor; hFGF-B: Human fibroblast growth factor-B; IGF-1: R3-insulin-like growth factor-1; NO: Nitric oxide; PI3K: Phosphatidylinositol 3-kinase; PPAR- γ : Peroxisome proliferator-activated receptor gamma; ROS: Reactive oxygen species; TCA: Trichloroacetic acid; TZD: Thiazolidinedione; VEGF: Vascular endothelial growth factor.

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Authors' contributions

LE and ÖE performed the experimental procedures, performed the statistical calculations, and contributed to results interpretation and discussion. TN, QZ and ÅS provided expertise in diabetes and endothelial dysfunction, the *in vitro* model, and conceived, designed and co-ordinated the research plan, respectively. LE, QZ and ÅS wrote the manuscript. LE, TN, QZ and ÅS contributed to discussion and edited the paper prior to submission. All authors read and approved the final manuscript.

Competing interests

Dr. Sjöholm has received research grants, and provided lectures, consultancies and expert testimony to several pharmaceutical companies

involved in diabetes care, such as GlaxoSmithKline, Takeda Pharmaceuticals North America, Schering-Plough, Novo-Nordisk, Eli Lilly, Novartis, Aventis, Sanofi-Synthelabo, Bristol-Myers, Squibb, Merck Sharp & Dohme, Pfizer, Boehringer-Ingelheim, Selena-Fournier, Roche Diagnostics, Astra-Zeneca, Bayer, Pharmacia, and Hässle Pharmaceuticals.

Dr. Sjöholm is also on the National and/or European Advisory Boards in Diabetes Care for Eli Lilly, Takeda Pharmaceuticals, Novartis, Sanofi-Aventis, Merck Sharp & Dohme, Boehringer-Ingelheim and Astra-Zeneca.

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