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Insulinotropic compounds decrease endothelial cell survival

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ABSTRACT

Objectives: Hyperglycemia induces damage of vascular endothelial cells leading to diabetic complications. We investigated the effects of insulinotropic compounds and elevated glucose on endothelial cells in the absence or presence of vascular endothelial growth factor (VEGF).
Results: Human umbilical vein endothelial cells (HUVECs) were treated with glibenclamide, repaglinide and insulinotropic imidazolines at high glucose concentration in the presence or absence of VEGF and viability, proliferation and nitric oxide production were measured. Hyperglycemia inhibited pro-survival effects of VEGF on endothelial cells. Glibenclamide and repaglinide decreased HUVEC viability at elevated glucose concentration in the absence but not in the presence of VEGF, without affecting HUVEC proliferation. Repaglinide also had some positive influence on HUVEC function elevating NO production in the presence of VEGF. Imidazolines showed different activities on endothelial cell survival. Efaroxan diminished HUVEC viability at elevated glucose concentration in the presence, however not in the absence of VEGF, while RX871024 decreased HUVEC survival regardless of the presence of VEGF.
Significance of the study: Our data demonstrate an important interplay between the actual insulinotropic compounds, VEGF and ambient glucose concentration affecting the survival of the vascular endothelial cells. Consequently, this interplay needs to be taken into consideration when designing novel oral antidiabetic compounds.

1. Introduction

Macrovascular and microvascular diseases are the major causes of morbidity and mortality in diabetic patients (Ding and Triggle, 2010). Normally, endothelial cells modulate vascular function and structure synthesizing and releasing vasoactive substances (Sena et al., 2013). Hyperglycemia which is a hallmark of diabetes exerts adverse effects on endothelial cells resulting in endothelial dysfunction, which is referred to as an imbalance in the release of vasodilating and vasoconstricting factors (Ding and Triggle, 2010). The most potent and abundant endothelium-derived relaxing factor is NO. Besides this, NO inhibits endothelial cell apoptosis and stimulates endothelial cell proliferation (Ahanchi et al., 2007; Sena et al., 2013). Under normal conditions, cumulative damage of endothelial cells is balanced by reparative processes. High glucose concentration inhibits endothelial cell NO production leading to vasoconstriction, decreased wound healing, thrombosis and increased white blood cell adhesion, accounting for the many known vascular complications in diabetes (Reusch and Draznin, 2007; Sena et al., 2013). Moreover, diabetes hyperglycemia, affecting many metabolic pathways in endothelial cells, induces endothelial cell death and inhibits endothelial cell migration and proliferation (Kowluru, 2005; Reusch and Draznin, 2007), thus disrupting the balance between endothelial cell damage and repair. Altogether this leads to damage of the vascular endothelial cell layer thus facilitating the formation of atherosclerotic lesions (Reusch and Draznin, 2007) and acellular capillaries (Kowluru, 2005).

VEGF is a main regulator of blood vessel growth under physiological and pathological circumstances. This angiogenic growth factor enhances endothelial cell proliferation, migration, differentiation, survival and NO production (Koch and Claesson-Welsh, 2012). However, the diabetic conditions affect VEGF expression, i.e., both plasma level of VEGF and its expression in the heart of diabetic patients are reduced (Chou et al., 2002), while in retina and glomeruli its expression is elevated (Chou et al., 2002; Nakagawa, 2007), suggesting that it participates in the progression of diabetic complications.

Insulinotropic compounds are widely used in the clinic for the treatment of diabetes. Currently used drugs close the K_{ATP} channels, the latter consisting of Kir6.2 and SUR1 subunits, in the plasma membrane of pancreatic β-cells leading to membrane depolarization and Ca^{2+} influx. The resulting increase in cytoplasmic free Ca^{2+} concentration, $[Ca^{2+}]_i$, induces insulin secretion. Endothelial cells as well possess K_{ATP} channels (Schnitzler et al., 2000; Yoshida et al., 2004) which, though consisting of a heteromultimeric complex of Kir6.1, Kir6.2 and SUR2B subunits, are also inhibited by currently used insulin secretagogues (Janigro et al., 1993; Katnik and Adams, 1997; Quast et al., 2004). K_{ATP} channels transduce changes in cell metabolism into changes in membrane potential,

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which is an important determinant of endothelial cell function. It has been shown in vivo that K_{ATP} channel activation leads to reduction in endothelial cell dysfunction in humans (Broadhead et al., 2004) and rats (Horinaka et al., 2001) and is accompanied by an increase in endothelial cell nitric oxide synthase expression (Horinaka et al., 2001). The K_{ATP} channel blocker glibenclamide, a currently used insulinotropic compound, abolished the observed effects (Horinaka et al., 2001; Broadhead et al., 2004).

In the present study, we investigated the effects of insulinotropic compounds on endothelial cells in the presence of a high glucose concentration, conditions mimicking those observed in diabetes. Specifically we investigated the effects of these compounds on NO production, proliferation and viability in the absence or presence of VEGF. For this investigation, two types of oral insulinotropic compounds were selected. The first type includes currently used antidiabetic drugs, second-generation sulfonylurea compound and glinide compound, exemplified by glibenclamide and repaglinide, respectively. The second type involves potential oral insulinotropic imidazolines, i.e., RX871024 and efaroxan. A part of this investigation was presented in abstract form (Zaitsev et al., 2012).

2. Material and methods

2.1. Materials and reagents

M199, penicillin, streptomycin and fetal calf serum were obtained from Gibco (Middlesex, UK). Endothelial cell growth supplement, heparin, glibenclamide and efaroxan were purchased from Sigma (St. Louis, MO, USA). RX871024 was obtained from Reckitt and Colman (UK) and repaglinide was obtained from Novo Nordisk Pharma (Bagsvaerd, Denmark). VEGF was purchased from PeproTech (London, UK). All other reagents were of analytical grade and were obtained from VWR International (West Chester, PA, USA).

2.2. Cell line and culture conditions

Human umbilical vein endothelial cells (HUVECs) were cultured in M199 culture medium containing 5.5 mM glucose supplemented with 20% (vol/vol) fetal calf serum, 75 μg/ml endothelial cell growth supplement (ECGS), 16 U/ml heparin, 50 U/ml penicillin, and 50 μg/ml streptomycin. Medium was changed to a fresh one with the same test substances every 48 h.

2.3. Cell viability assay

HUVEC viability was detected using CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega (Madison, WI, USA), according to the manufacturer's instructions. The method is based on spectrophotometric detection of a colored formazan product converted from an MTS tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] by NADPH or NADH in metabolically active cells. Subsequent to incubation for 9 days with test substances, cells were washed three times with fresh culture medium and then incubated in fresh medium for 2 h. Assays were performed by adding 20 μl of the CellTiter 96® AQueous One Solution Reagent to culture wells (100 μl culture medium/well), incubating for 1–4 h and then recording absorbance at 490 nm with a 96-well plate reader.

2.4. Assessment of HUVEC proliferation

Assessment of HUVEC proliferation was performed using BrdU Cell Proliferation Assay, Calbiochem (SanDiego, CA, USA). HUVECs were exposed to substances for 90 h and BrdU incorporated into the DNA of dividing cells was immunodetected according to the manufacturer's instructions.

2.5. Assay of nitrite production

HUVEC nitrite production was determined with Griess reaction. After incubation of the cells with test substances for 40 h, culture medium was withdrawn and centrifuged for 2 min at 1500*g*. 100 μl samples of supernatant were transferred to a 96-well plate and mixed with 50 μl of Griess reagent (Alexis Corporation Carlsbad, CA, USA), as previously described (Zaitsev et al., 2001; Zaitseva et al., 2006). The reaction was carried out for 15 min at room temperature. Nitrite production was determined at 540 nm absorbance with reference at 620 nm in a 96-well plate reader. For calibration of data, a standard curve for $NaNO_2$ in M199 was established in each assay. The results are expressed as μM of NO_2^-.

2.6. Statistical analysis

All results are expressed as means ± SEM for the indicated number of experiments with 5–6 observations per each condition. Statistical significance between means was assessed by one-way or two way ANOVA and Student's t-test for unpaired values with significance set to $P < 0.05$.

3. Results

3.1. High glucose decreases HUVEC viability, proliferation and NO production in the presence of VEGF

Well-known deleterious effects of elevated glucose on endothelial cells are the induction of apoptosis, the reduction of NO production and proliferation (Reusch and Draznin, 2007). Under normal conditions, angiogenic cytokine VEGF enhances endothelial cell survival, NO production and proliferation (Koch and Claesson-Welsh, 2012). We aimed to investigate the effects of insulinotropic compounds on endothelial cells under hyperglycemic conditions in the presence and absence of VEGF. Therefore, we first investigated whether VEGF can affect endothelial cells in the presence of high glucose concentration. As expected, 25 mM glucose diminished HUVEC viability after 9 days incubation (Fig. 1A) when compared with a normal glucose concentration of 5.5 mM. Moreover, high glucose decreased HUVEC proliferation to 88 ± 4% ($P < 0.05$, $n = 6$, unpaired Student's t-test) after 90 h incubation compared to normal glucose. Reduction of HUVEC viability and proliferation was in line with a decrease in NO production induced by elevated glucose (Fig. 1B). The presence of 100 ng/ml VEGF in culture media elevated HUVEC viability at 5.5 mM glucose (Fig. 1A), but had no effect on high glucose induced reduction in HUVEC viability (Fig. 1A). Moreover, elevated glucose inhibited HUVEC NO production even in the presence of VEGF (Fig. 1B). Similarly, despite the addition of the angiogenic growth factor, 25 mM glucose still reduced HUVEC proliferation to 84 ± 4% compared to 5.5 mM glucose and VEGF ($P < 0.05$, $n = 6$, unpaired Student's t-test). These observations suggest that hyperglycemia inhibits pro-survival effects of VEGF on endothelial cells.

3.2. Insulinotropic imidazolines can cause HUVEC death at elevated glucose concentration

It was previously shown that some compounds with imidazoline moiety, for instance phentolamine, can affect vascular smooth muscle K_{ATP} channels (Okumura et al., 1992). Vascular myocytes contain K_{ATP} channels consisting of Kir6.2 and SUR1, which resemble endothelial K_{ATP} channels (Quast et al., 2004). Insulinotropic imidazolines were shown to inhibit the Kir6.2 subunit of the K_{ATP} channels (Proks and Ashcroft, 1997; Efanov et al., 2001). Taking this into consideration, we investigated whether insulinotropic imidazolines can affect endothelial cells under diabetic conditions. For these purposes, HUVECs were exposed to 50 μM of test substances (RX871024 or efaroxan) at 25 mM glucose in the presence or absence of 100 ng/ml VEGF and endothelial cell function and survival were monitored by measuring HUVEC NO