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eNOS, COX-2, and prostacyclin production are impaired in endothelial cells from diabetics

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Abstract

The vascular endothelium is a well-recognized target of damage for factors leading to increased cardiovascular risk. Among the agents playing an important role in cardiovascular homeostasis, nitric oxide and prostacyclin represent key markers of endothelial integrity. In the present work, we report for the first time the reduced expression of both endothelial nitric oxide synthase and cyclooxygenase-2 (COX-2) proteins, as well as decreased prostacyclin production, in unstimulated human endothelial cells from insulin-dependent diabetic mothers when compared to cells from non-diabetic, control subjects. According to a major role of COX-2 as a source of prostacyclin production even in unstimulated endothelial cells, prostacyclin production was concentration-dependently inhibited by the selective COX-2 inhibitor SC236. Overall, our results suggest a possible link between reduced endothelial COX-2 and NO-synthase expression and the increased risk of cardiovascular diseases affecting diabetic patients, and point to the use of endothelial cells from diabetic patients as a tool for investigating early dysfunction in pathological endothelium.

Keywords: eNOS; COX-2; Prostacyclin; Insulin-dependent diabetes; Endothelial cells

Endothelial dysfunction is well recognized as a common target of cardiovascular risk factors, and refers to the broad alterations in endothelial phenotype that contribute to the development and clinical expression of atherosclerosis [1]. The risk for all forms of cardiovascular disease is substantially increased with type 1 and type 2 diabetes [2]. Suitable models of human "pathological endothelium" may greatly expand knowledge of the mechanisms responsible for initiation and progression of endothelial dysfunction.

Cyclooxygenase-2 (COX-2) and endothelial nitric oxide synthase (eNOS) are primarily expressed in endothelial cells and are considered as important regulators of vascular function. Under normal conditions, laminar flow induces COX-2 expression and synthesis of PGI_2 which in turn stimulates eNOS activity [3]; the net effects of these actions are to provide optimal control of vascular tone and homeostasis, and to prevent platelet activation and aggregation [4]. Human and experimental models of diabetes have showed endothelial dysfunction and atherogenic predisposition; however, to date, a direct profiling of enzymes and mediators in endothelial cells from diabetic patients is still lacking.

Exposure to a diabetic environment in utero is associated with increased occurrence of impaired glucose tolerance and defective insulin secretory response in adult offspring of type 1 diabetic mothers, regardless of genetic predisposition to type 2 diabetes [5] resulting in impaired foetal birth weight, strictly related to altered nutrient availability [6,7]. Disproportionate foetal growth may later lead to major cardiovascular events in agreement with the foetal origins of coronary heart disease [8]. Based on these considerations, we tested the hypothesis that umbilical vein endothelial cells from insulin-dependent diabetic mothers may already exhibit phenotype alterations.

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Materials and methods

Cell culture. Endothelial cells were obtained from type 1 insulin-dependent diabetic (dHUVEC) and healthy, non-diabetic mothers (HUVEC) as previously described [9]. Cells were grown in medium 199 (M199) supplemented with 15% FCS (Euroclone), gentamicin (40 μg/ml, Gibco), endothelial cell growth factor (25 μg/ml, Sigma), and heparin (100 μg/ml, Sigma). For experiments, cells were incubated for 6 h with medium 199 supplemented with 5% FCS. In selected experiments, SC236 was added for the same period of time.

Determination of PGI_2 production. After incubation, the culture medium was collected and centrifuged at 12,000 rpm for 5 min. PGI_2 was measured as its stable hydrolysis product 6-keto-$PGF_{1\alpha}$ with an ELISA kit (Cayman Chemical) according to the manufacturer's instructions. Results were expressed as ng PGI_2/mg cell protein.

Western blot analysis. At the end of incubations, cells were harvested in lysis buffer and after quantization by Lowry's method, 15 μg cell protein was loaded onto 10% SDS–acrylamide gels. At the end of the run, proteins were transferred to a nitrocellulose membrane and incubated with polyclonal antibodies against COX-2 (1:400, Cayman) and eNOS (1:1000, Santa Cruz) overnight, and then with suitable peroxidase-conjugated secondary antibodies for 1 h. Proteins were detected by chemiluminescence (Amersham Biosciences). Loading control was performed using actin immunodetection.

Statistical analysis. Data were obtained from 4 (insulin-dependent diabetic patients) to 19 (non-diabetic patients) independent experiments, each value representing mean ± SEM of duplicate or triplicate determinations. Comparison between groups was performed by ANOVA or Mann–Whitney test for non-parametric data. Values of $p < 0.05$ were considered statistically significant.

Results and discussion

The results provide evidence, for the first time, that unstimulated dHUVEC show reduced expression of endothelial nitric oxide synthase (eNOS) (−20%, Fig. 1A) and COX-2 (−40%, Fig. 1B) when compared to HUVEC. Accordingly, a significantly reduced production of prostacyclin (PGI_2), measured as its stable metabolite 6-keto $PGF_{1\alpha}$, was observed in resting dHUVEC when compared to HUVEC (2.6 ± 0.64 vs 8.0 ± 1.1 ng/mg prot, Fig. 1C). This effect was maintained after several cell duplications in culture (data not shown). COX-2 and eNOS represent key markers of endothelial integrity [1,4] and their respective products PGI_2 and NO, sharing antiadhesive, antithrombotic, and antiproliferative properties, play a major role in the maintenance of vascular homeostasis. A cross talk between the two pathways is operative under physiological conditions [4,10], and our data show that the diabetic condition negatively affects both systems. It is also of note that the reduced expression of COX-2 observed in dHUVEC appears to proportionally affect PGI_2 levels, suggesting that the production of such eicosanoids in unstimulated cells is mainly dependent on the inducible isoform of COX rather than on the constitutive isoform COX-1. Accordingly, we found a significantly reduced basal PGI_2 formation by HUVEC upon treatment with SC236 (Fig. 2), an early structural lead of sulphonamide derivatives which differs from celecoxib by a chloride in place of a methyl group at position 4 in the aromatic ring [11]. Indeed, SC236 almost completely abolished basal PGI_2 synthesis observed in unstimulated HUVEC, with an EC_{50} value of 10 nM (Fig. 2). The inhibition of PGI_2 production by SC236 in unstimulated HUVEC was

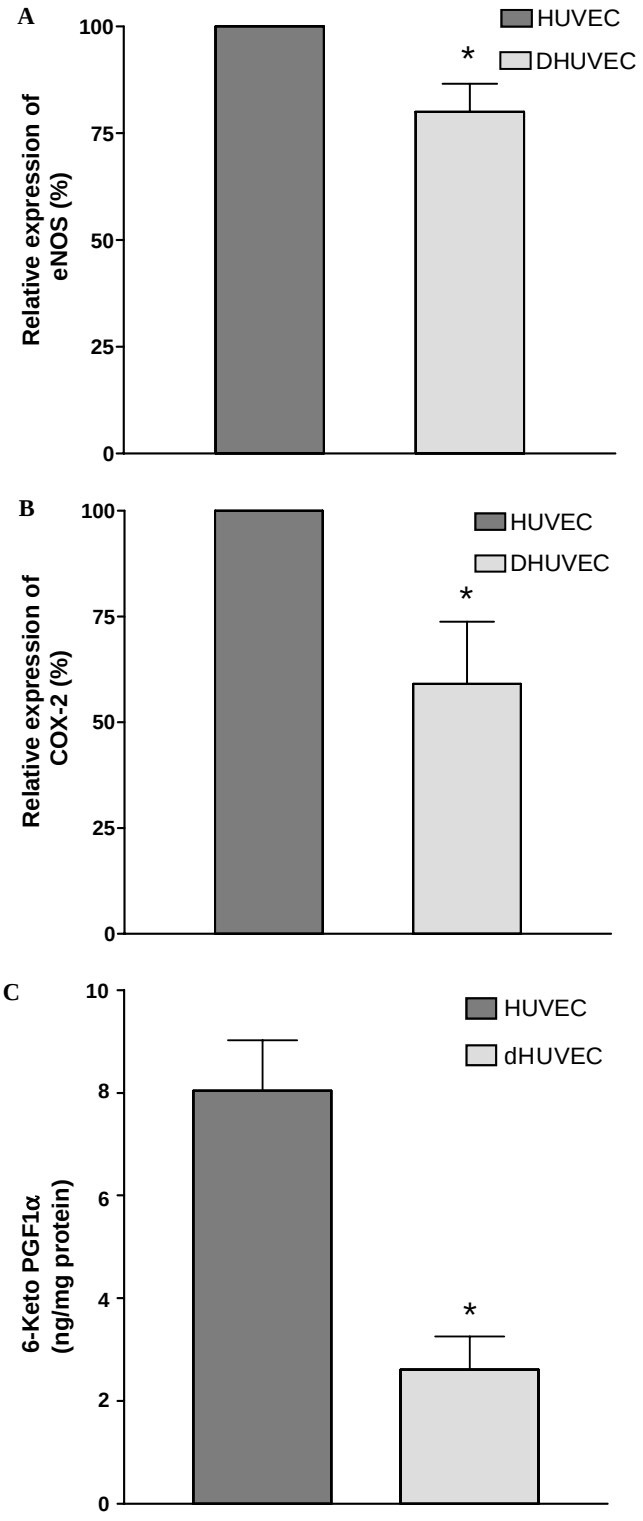

Fig. 1. COX-2 (A) and eNOS (B) protein expression and prostacyclin production (C) in HUVEC from insulin-dependent diabetic mothers compared to non-diabetic, control subjects. Data are expressed as means ± SE of 4–20 independent determinations. *$p < 0.05$ vs. control (ANOVA, COX-2 and eNOS; Mann–Whitney test, 6-keto-$PGF_{1\alpha}$).

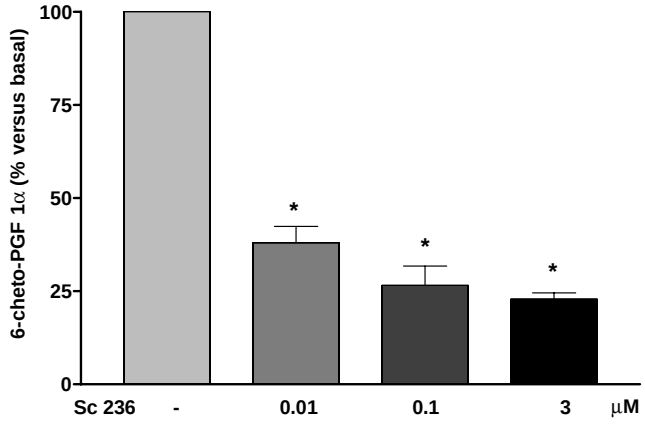

Fig. 2. Effects of the selective COX-2 inhibitor SC236 on the basal formation of prostacyclin. Basal production was 6.6 +/− 1.8 ng/mg cell protein. Data are expressed as means ± SE of five independent determinations. *$p < 0.05$ vs. control (ANOVA).

concentration-dependent in the range 0.01–3 μM. Since peak plasma concentrations of different COX-2 inhibitors range from 147 to 842 μg/ml, the effects of SC236 herein reported fall within a concentration range that is most likely relevant in vivo. At the highest concentration tested (3 μM), treatment with SC236 decreased basal PGI_2 production by about 75%, which is very well in line with the reduction in urinary excretion of 2,3-dinor-6-keto $PGF_{1\alpha}$ detected in healthy subjects after dosing with 400 and 800 mg celecoxib [12]. This finding supports the current view that COX-2, and not COX-1, is the major PGI_2-generating enzyme in vascular endothelium.

In conclusion, we have shown that endothelial cells from insulin-dependent diabetic patients feature a down-regulated expression of COX-2 and eNOS enzyme proteins, whose products strategically cooperate in the protection of the vessel wall. These findings may have clinical relevance given the critical role of the endothelium in the development of cardiovascular disease whereby diabetes represents a significant risk factor.

Moreover, to the best of our knowledge, our findings provide the first in vitro demonstration of a significant contribution of COX-2 to the unstimulated production of PGI_2 in the vascular endothelium, in line with the potential mechanism of the reported cardiovascular toxicity of COX-2 inhibitors in vivo.

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