Hyperglycaemia, reperfusion injury and menadione cause identical damage to the mitochondrial DNA of cultured vascular endothelial cells

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Hyperglycaemia increases single strand breaks in the DNA of cultured vascular endothelial cells ex vivo and induces an increase in DNA repair [1]. In vivo it accelerates atherosclerosis and can lead to reduced blood flow or ischaemia which precedes reperfusion injury [2]. We report that hyperglycaemia, reperfusion injury and menadione (an oxygen radical generator in endothelial cells) induce the same 4977 bp deletion in the mitochondrial DNA (mtDNA) of vascular endothelial cells. This result was confirmed by sequencing studies to be reported elsewhere. Flanking sequences were shown to include a pair of 13 base pair (bp) repeats (ACCTCCCTCACCA), only one of which was removed by deletion. We have shown that diabetic patients with persistent hyperglycaemia (HbA1C > 9% of total haemoglobin) also harbour this deletion in the mtDNA of their endothelial cells in vivo [3].

Receptors have recently been identified on the surface of both endothelial cells and macrophages which bind proteins modified by non-enzymatic glycation, termed advanced glycosylation end products (AGE products) [4]. These scavenger receptors remove AGE-proteins from the circulation [5] and, under pathological conditions, may, under pathological conditions, be responsible for the oxidative damage to endothelial cells which leads to the vascular complications of diabetes. To model ex vivo the effects of hyperglycaemia in diabetics, we found it necessary to treat cultured human umbilical vein endothelial cells (HUVEC) with both D-glucose (15 to 30 mM) and AGE-albumin (0.6 to 6 mg/ml). AGE-albumin was prepared as previously described [5]. HUVEC were isolated [6] and cultured in 5 ml of medium 199 supplemented with 20% (v/v) fetal calf serum, 2 mM L-glutamine, 50 units heparin per ml medium, 30 µg/ml of endothelial cell growth factor, 100 units/ml penicillin and, 0.1 mg/ml streptomycin. The cells were routinely seeded into 30 mm petri dishes at densities of 5 x 10⁵ cells. Following attachment the medium was supplemented with dialysed AGE-albumin to final concentrations of 6 or 0.6 mg/ml. The cells were grown for 4 days at 37°C in an atmosphere of 5% (v/v) CO₂ in air. Total DNA was isolated [7] and subjected to PCR using mtDNA primers DEL1 (np 8345-8364) and DEL2 (np 1327-13808, numbering according to [8]). The results shown in Fig 1A demonstrate that cells accumulate a 4977 bp mtDNA deletion when the medium is supplemented with both 6 mg/ml AGE-albumin and 30 mM D-glucose, but not with either dialysed AGE-albumin or D-glucose alone. A lower level of deletion could also be induced by (a) 0.6 mg/ml AGE-albumin plus 30 mM D-glucose or (b) 6 mg/ml AGE-albumin plus 15 mM D-glucose, but not by (c) 6 mg/ml AGE-albumin plus 5 mM D-glucose or (d) 6 mg/ml AGE-albumin plus 30 mM D-glucose (results not shown). In a related experiment, HUVE cells were grown in the presence of the lipophilic quinone, menadione. This compound is known to generate superoxide in endothelial cells when the semiquinone form reacts with molecular oxygen [9]. Treatment of cells with 20 µM or 2 µM menadione was found to generate the same 4977 bp mtDNA deletion (Fig 1B). The 4977bp mtDNA deletion was also induced when endothelial cells were subjected to reperfusion injury in the form of 45 minutes anoxia followed by reoxygenation of the medium and further culturing for up to 4 days (results not shown). The effect of 50 µg/ml α-tocopherol (Vitamin E) on HUVE cells, grown for 4 days in the presence of 6 mg/ml AGE-albumin and 30 mM D-glucose, was to prevent the induction of the 4977 bp mtDNA deletion (Fig 1C).

In order to determine the percentage of mtDNA molecules deleted in HUVE cells following treatment with both 6 mg/ml AGE-albumin and 30 mM D-glucose, quantitative PCR was performed on restricted DNA isolated from treated cells [10] (Fig 2). Separate aliquots of DNA from 5 x 10⁵ cells were subjected to PCR using either (a) primers DEL1 and DEL2, or (b) primers TOT1 (np 3108-3127) and TOT2 (np 3649-3650). The latter primers were chosen from a region of the mtDNA which is rarely deleted. In Fig 2, the intensity of each PCR band, as measured by laser densitometry, is plotted against cell number. The percentage of total mtDNA with the 4977 bp deletion was calculated [10] to be approximately 5% of mtDNA in HUVE cells treated with 6 mg/ml AGE-albumin and 30 mM D-glucose for 4 days.

The observation that Vitamin E (a free radical quencher) prevents the hyperglycaemia-induced deletion, suggests that a free radical mechanism might be involved. In studies to be reported elsewhere, we have observed that, as the number of mtDNA molecules harbouring the 4977 bp deletion increases in cultured endothelial cells ex vivo, they stop dividing and detach from their substratum. We have shown that endothelial cells in vivo in diabetic patients with persistent hyperglycaemia (HbA1C > 9%) also appear to detach from their basement membrane as they accumulate the 4977 bp mtDNA deletion [3]. In the case of reperfusion- and menadione-induced injury, there can be little doubt that oxygen radicals are involved in the induction of the 4977 bp deletion. We have recently shown that H₂O₂ can also induce this deletion. The mechanism whereby oxygen radicals (generated in the cell) induce the 4977 bp deletion may involve an illegitimate recombination between the two 13 bp repeats which flank the deleted sequence. A similar mechanism appears to operate in the DEL system [11] (S. cerevisiae diploid strain RS112) which, like cultured human endothelial cells, can be used to measure the frequency of a particular DNA deletion produced by an illegitimate intrachromosomal recombination/ slippage mechanism. In conclusion, a variety of results, including H₂O₂, menadione and reperfusion injury can generate a 4977 bp deletion in the mtDNA of vascular endothelial cells by a mechanism which involves oxygen radicals. The hyperglycaemic insult is more complex since to generate the 4977 bp deletion over a short period (4 days), one needs both AGE-albumin (0.6 to 6 mg/ml) and D-glucose (15 to 30 mM). D-glucose cannot be replaced by L-glucose or normoglycaemic D-glucose (5 mM).

![Figure 1](image1)

**Figure 1.** Accumulation of the 4977 bp deletion in mtDNA of HUVE cells (A) Lane 1: 1 kb BRL ladder (size markers). PCR was performed on total DNA isolated from 5 x 10⁵ HUVE cells using primers DEL1 and DEL2. Cells were treated as follows: Lane 2: dialysed AGE-albumin (6 mg/ml). Lane 3: 0.6 mg/ml dialysed AGE-albumin. Lane 4: dialysed AGE-albumin (6 mg/ml) together with 30 mM D-glucose. Lane 5: dialysed AGE-albumin (0.6 mg/ml) and 30 mM D-glucose. (B) Accumulation of the 4977 bp deletion in mtDNA of HUVE cells following treatment with 20 µM (Lane 2) and 2 µM (Lane 3) menadione. Lane 1: 1 kb BRL ladder (size markers). PCR was performed on 5 x 10⁵ cells using primers DEL1 and DEL2. (C) Effect of 50 µg/ml α-tocopherol on the level of the 4977 bp mtDNA deletion in HUVE cells treated with AGE-albumin and/or D-glucose. Lane 1: 1 kb BRL ladder (size markers). PCR was performed on total DNA isolated from 4 x 10⁵ HUVE cells using primers DEL1 and DEL2. Cells were treated with: Lane 2: 6 mg/ml AGE-albumin (dialysed) only. Lane 3: dialysed AGE-albumin (6 mg/ml) and 30 mM D-glucose. Lane 4: 6 mg/ml dialysed AGE-albumin and 30 mM D-glucose in the presence of 50 µg/ml α-tocopherol.

3. Egawhary, D.N., Swoboda, B.P., Chen, J.A., Vince, F.P. (see TRANSACTION 17)
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