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Advanced glycation endproducts induce a proliferative response in vascular smooth muscle cells via altered calcium signaling

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ABSTRACT

Advanced glycation endproducts (AGEs) are proteins that accumulate in the plasma of diabetics as a result of increased glucose concentrations and are closely linked with vascular disease. The mechanisms involved are still not clear. The aim of this study was to investigate whether AGE-induced changes in calcium (Ca^{2+}) homeostasis could contribute to these mechanisms. Cultured porcine coronary artery vascular smooth muscle (VSM) cells were preincubated with glycated albumin for 96 h. The sphingosine 1-phosphate (S1P)-induced intracellular Ca^{2+} increase, although not increased in amplitude, was significantly prolonged in cells preincubated with glycated albumin. Intracellular Ca^{2+} imaging and electrophysiological recording of ion channel currents following release of caged Ca^{2+} indicated that this prolonged Ca^{2+} rise occurred predominantly via changes in Ca^{2+}-induced Ca^{2+} release. Preincubation with glycated albumin also resulted in a threefold increase in expression of the receptor for AGE. As a consequence of the prolonged intracellular Ca^{2+} rise following preincubation with glycated albumin, the S1P-induced activation of the Ca^{2+}-dependent phosphatase, calcineurin (CaN) was increased. This resulted in increased S1P-induced activation of the Ca^{2+}-dependent transcription factor, nuclear factor of activated T cells (NFATc). BrdU incorporation in VSM cells was increased in cells preincubated with glycated albumin and was inhibited by the CaN inhibitor, cyclosporin A. In conclusion, AGE can induce VSM proliferation via a prolonged agonist-induced Ca^{2+} increase leading to increased activation of CaN and subsequently NFATc. This mechanism may contribute to pathogenesis of vascular disease in diabetes mellitus.

1. Introduction

Diabetes mellitus is a disease characterized by chronic hyperglycaemia due to a deficiency in insulin action. Hyperglycaemia is the primary factor that initiates vascular complications associated with diabetes such as nephropathy, retinopathy and the development of atherosclerosis [1–3]. Based on experimental evidence, several hypotheses have been suggested which could link hyperglycaemia to diabetic vascular complications [4–9]. In recent years, much research has focussed on a mechanism intimately linked with vascular complications, and is also the direct result of hyperglycaemia. Evidence now suggests that many of the vascular complications of diabetes may be mediated by advanced glycation endproducts (AGEs) [10–12]. AGEs are modifications of proteins and lipids that become nonenzymatically glycated and oxidized after contact with sugars [13]. The AGE formation begins with covalent binding of aldehyde or ketone groups of

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reducing sugars, such as glucose, to free amino acid groups of proteins. This undergoes rearrangement to ketoamines (Amadori's product) with highly reactive carbonyl groups (formed as a result of carbonyl stress) [14,15]. These intermediates accumulate and react with various functional protein groups leading to the formation of stable AGE compounds [15]. AGEs, once formed, are mostly irreversible. A crucial factor in the formation of AGEs is the degree of hyperglycaemia [16,17]. Therefore, in diabetes, the intracellular formation of AGEs is greatly increased [16–18]. The importance of AGE in the pathogenesis of diabetic vascular complications is demonstrated by several studies showing AGE inhibitors can reduce microvascular damage in retina, kidney and nerve [19,20]. AGEs are also enriched in atherosclerotic lesions in both human diabetes [21] and in animal models of diabetes [22]. In addition, AGEs can induce vascular smooth muscle (VSM) cell proliferation *in vitro* [23,24] although the mechanisms of this are not clear.

Recently, the potential pathophysiological relevance of circulating AGEs was indicated by the discovery of the receptor for advanced glycation endproducts (RAGE) [25]. RAGE is a multi-ligand member of the immunoglobulin superfamily containing a single transmembrane spanning domain and is expressed in a variety of cell types, including endothelial and VSM cells [26]. Apart from AGEs, other endogenous ligands for RAGE include amphoterin and S100/calgranulin proteins [27]. In addition to the full-length form of RAGE, truncated forms have also been identified with both C- and N-terminal truncations [28]. Only C-terminal truncations and full-length RAGE forms can bind AGEs [28]. RAGE is typically expressed at low levels in cells and may be upregulated in certain conditions [25]. Intracellular pathways stimulated following RAGE engagement with ligand are predominantly those involved in inflammatory responses [29]. RAGE signaling results in activation of a number of pathways, such as mitogen-activated protein (MAP) kinases (extracellular regulated signal kinase (ERK)1/2 and p38MAP kinase), and the small GTPases CDC42, Rac and Ras [30]. In endothelial cells this can result in the activation of the proinflammatory transcription factor, nuclear factor κB (NFκB) [31]. In VSM cells, the RAGE-activated signaling pathways are less clear although activation of Ras, ERK1/2 and NFκB has been previously demonstrated [24,32]. Key studies have now revealed the importance of RAGE in the pathogenesis of atherosclerosis in diabetes [21]. In animal models such as apolipoprotein E knockout mice treated with streptozotocin, blockade of RAGE significantly reduced the formation of atherosclerotic lesions [33]. RAGE knockout transgenic mice also displayed a decreased arterial restenosis following arterial injury compared to wild type mice [34].

An important factor regulating VSM cell phenotype is Ca^{2+}-dependent signaling. Ca^{2+}-dependent transcription factors can play a critical role in modulation of VSM phenotype [35–37]. In cardiac myocytes, AGEs have been reported to alter Ca^{2+} homeostasis [38] although this has not been examined in VSM cells. In the current study the effects of glycated albumin (an AGE found *in vivo*) on agonist-induced Ca^{2+} homeostasis were examined. Preincubation with AGEs for 96 h resulted in a prolonged increase in agonist-induced intracellular Ca^{2+} due, in part, to increased Ca^{2+}-induced Ca^{2+} release (CICR). This leads to increased activation of the Ca^{2+}-dependent phosphatase, calcineurin (CaN). This subsequently results in an activation of the Ca^{2+}-dependent transcription factor, nuclear factor of activated T-cells (NFATc) and increases cell proliferation.

2. Materials and methods

2.1. Primary cell culture

Adult male pig hearts were obtained from a local abattoir and immediately placed in Hanks balanced salt solution. The left descending coronary artery was dissected, connective tissue removed and the endothelium denuded. Arteries were incubated in serum-free Dulbecco's modified Eagle's medium (DMEM) containing 1 mg/ml collagenase (type II), 0.2 mg/ml elastase (type IV) and 50 μg/ml soybean trypsin inhibitor at 37 °C. The tissue was titurated every 30 min until complete dispersal had occurred (3–4 h), transferred to an 80 cm^2 tissue culture flask containing DMEM, 20% foetal bovine serum (FBS), 2 mM L-glutamine, penicillin (10,000 units/ml) and streptomycin (10 mg/ml) and incubated at 37 °C, in a humidified 5% CO_2 atmosphere. After 24 h, the medium was removed and cells transferred to fresh DMEM, 10% FBS, 2 mM L-glutamine, penicillin (10,000 units/ml) and streptomycin (10 mg/ml). Cells were used for experiments between passages 4 and 8. The *n* values given in Figs. 1–3 represent individual measurements from primary cultured single cells derived from at least three different animals.

2.2. Imaging of $[Ca^{2+}]_i$

Cultured VSM cells were grown on glass-bottomed dishes and serum-starved 24 h before use. Cells were loaded with 4 μmol/l Fura-2 AM for 30 min in a solution containing (in mmol/l): 130 NaCl, 5.6 KCl, 1 $MgCl_2$, 1.7 $CaCl_2$, 11 Glucose, 10 HEPES (pH 7.4) followed by a 20 min de-esterification period. A Zeiss Axiovert 200 inverted microscope, equipped with a cooled CCD camera (Photometrics, Tucson, AZ) and a polychromatic illumination system (T.I.L.L. Photonics, Gräfelfing, Germany), was used to capture fluorescence images with excitations at 340 and 380 nm. The ratio of the fluorescence intensity between the pair of frames (FR340/380) was calculated after background subtraction. The Metafluor software (Molecular Devices, PA) controlled the illuminator and camera, and performed image ratio measurements and analysis. Results are expressed as F340/380 ratio. Experiments were carried out at room temperature (22–24 °C).

2.3. Electrophysiology

The whole cell variant of the patch clamp technique was used to record ion channel currents from cultured VSM cells. Patch pipettes (resistances 5–9 MΩ) were filled with a KCl-based solution containing (in mmol/l) 140 KCl, 0.1 $CaCl_2$, 2 $MgCl_2$, 5 EGTA, 10 HEPES, 2 ATP (310–320 mOsm/l and pH 7.2). Recordings were made with extracellular solutions that contained (in mmol/l) 3 KCl, 0.6 $MgCl_2$, 2 $CaCl_2$, 1 $NaHCO_3$, 10 HEPES, 5 glucose and 130 NaSCN (310–320 mOsm/l and pH