

## Effects of advanced glycosylation end products on proliferation and cytosolic free calcium in cultured rat aortic smooth muscle cells

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**KEY WORDS** advanced glycosylation end products; calcium; vascular smooth muscle; cultured cells; diabetic angiopathies; atherosclerosis; thymidine; leucine

**AIM:** To study the effects of advanced glycosylation end products (AGEP) on aortic smooth muscle cell (ASMC) proliferation and its relationship with cytosolic free calcium ( $[Ca^{2+}]_i$ ). **METHODS:** The effects of AGEP modified bovine serum albumin (AGEP-BSA) on the incorporation of  $[^3H]$ TdR and  $[^3H]$ Leu into cultured ASMC were observed. The  $[Ca^{2+}]_i$  of cultured ASMC was determined with Fura 2-AM. **RESULTS:** AGEP-BSA stimulated the incorporation of  $[^3H]$ TdR and  $[^3H]$ Leu into ASMC in concentration and time-dependent manners ( $P < 0.01$ ), especially  $[^3H]$ TdR. In AGEP 200  $mg \cdot L^{-1}$  group, after 12-h exposure, the incorporation of  $[^3H]$ TdR obviously increased, but DNA synthesis was concentration-dependently decreased in AGEP 300 - 400  $mg \cdot L^{-1}$  groups. The peak incorporation of  $[^3H]$ TdR was 10 times vs control ( $P < 0.01$ ). The  $[Ca^{2+}]_i$  of ASMC incubated with AGEP-BSA for 40 min was increased from control groups  $121 \pm 4$  to  $492 \pm 20$   $nmol \cdot L^{-1}$  ( $P < 0.01$ ).  $[Ca^{2+}]_i$  induced by AGEP was elevated with the concentration and incubating time of glucose with BSA.  $[Ca^{2+}]_i$  in BSA incubated with glucose 80  $mmol \cdot L^{-1}$  for 12 wk was  $580 \pm 37$   $nmol \cdot L^{-1}$  ( $P < 0.01$ ). **CONCLUSION:** AGEP stimulated proliferation of ASMC associated with the elevation of  $[Ca^{2+}]_i$ .

The primary causal factor for the development of most diabetic complications is prolonged exposure to hyperglycemia<sup>[1]</sup>. The chemical interaction of glucose with proteins formed advanced glycosylation end products (AGEP), which may account for numerous

feature of diabetic complications and accelerated atherosclerosis<sup>[2]</sup>. AGEP exists in atherosclerotic lesions of human aorta by immunohistochemical and ultrastructural method<sup>[3]</sup>. AGEP have atherogenic properties, such as transendothelial chemotaxis of monocytes, oncogene expression, cytokine secretion, and platelet aggregation. Abnormal proliferation of vascular smooth muscle cells has a fundamental role in the atherogenesis which is enhanced by some growth factors/cytokines, hyperlipidemia, and hyperglycemia<sup>[4]</sup>. Cytosolic free calcium ( $[Ca^{2+}]_i$ ) is a common signal transduction element and may be closely involved with multiple cell response, especially for the proliferation of ASMC. The goal of this study was to investigate the effects of AGEP on proliferation of ASMC and its relationship with  $[Ca^{2+}]_i$ .

### MATERIALS AND METHODS

**Rats** Sprague-Dawley rats ( $\delta$ , adults,  $n = 25$ ) weighing  $196 \pm 14$  g, were provided by the Laboratory Animal Center of Nanjing Railway Medical College (No 95036).

**Reagents** Dulbecco's modified Eagle's medium (DMEM, Gibco product). Fura 2-AM (Sigma).  $[^3H]$ TdR and  $[^3H]$ Leu were purchased from Beijing Institute of Nuclear Research. Scintillation liquid was xylene containing 0.3 % diphenyloxazole and 0.003 % 1,4-di-[2-(5-phenyloxazolyl)]-benzene, POPOP. Other reagents were of AR.

**Cell culture** ASMC were cultured according to Kirstein<sup>[4]</sup>. All experiments were performed on the 3rd - 4th passages of ASMC isolated from Sprague-Dawley rat aorta.

**AGEP-BSA** AGEP-BSA was prepared by incubating BSA in phosphate-buffered saline (PBS) with glucose 5, 20, 50, and 80  $mmol \cdot L^{-1}$  at 37 °C in the presence of edetic acid 0.5  $mmol \cdot L^{-1}$ . Fluorescence spectra of AGEP-BSA was expressed by arbitrary fluorescence unit (AFU).

**$[Ca^{2+}]_i$**  Cells  $1 \times 10^6$  were seeded on a microscope cover slip (25 mm in diameter, Nuhclow, Denmark) in DMEM. After 12-h incubation, AGEP-BSA or BSA was added. The cells were incubated for 40 min in DMEM containing Fura 2-AM 5  $\mu mol \cdot L^{-1}$  at 37 °C in a dark compartment. The cells were washed thrice with a buffer medium ( $CaCl_2$  1.3  $mmol \cdot L^{-1}$ , pH 7.4) and kept in the same buffer. After 5 min, the coverslip was placed in an 1.0-mL Sykes-Moore chamber on an inverse

<sup>1</sup> Project supported by the National Natural Science Foundation of China, No 39470314.

<sup>2</sup> Pm: 86-25-331-8787, ext 3103.

Received 1996-07-08

Accepted 1997-05-29

phase-contrast microscope. Fluorescence was made<sup>[5]</sup>, using a DM 3000 spectrofluorometer (Hitachi).  $[Ca^{2+}]_i = K_d(R - R_{min}) / (R_{max} - R)$  nmol · L<sup>-1</sup>. The  $R_{max}$  and  $R_{min}$  were determined by Triton-100 and egtazic acid, respectively.

**DNA and protein synthesis** DNA and protein synthesis were assessed by measuring the incorporation rates of [<sup>3</sup>H]TdR and [<sup>3</sup>H]Leu<sup>[6]</sup>. ASMC were seeded at a density of  $1 \times 10^6$  cells/well in 24-well plate (Nuhclow, Denmark) and allowed to attach overnight. The medium containing standard 15 % fetal bovine serum (FBS) was replaced by medium containing 0.4 % FBS (vol/vol) for 48 h to arrest the cells in G<sub>0</sub> phase of the cell cycle. After 12-h incubation, AGEPE-BSA was added, and for the control groups, an equal amount of BSA was added. Both final [<sup>3</sup>H]TdR and [<sup>3</sup>H]Leu concentrations were 37 MBq · L<sup>-1</sup>. At the end of culture, the cells were treated with 0.1 % trypsin and harvested onto glass fiber filters (Hongguang Factory, Shanghai), which were washed with trichloroacetic acid 0.6 mol · L<sup>-1</sup>, and rinsed with 0.9 % NaCl. The precipitates were decolorized with ethanol, and stored at 105 °C. The radioactivities (Bq) were counted in a Packard 4000 liquid scintillation counter (Packard, USA).

**Data analysis** Data were expressed as  $\bar{x} \pm s$  and compared by ANOVA.

## RESULTS

**Fluorescence spectra during BSA incubated with glucose** AFU increased with incubation time and glucose concentrations (Tab 1).

**Tab 1. Fluorescence spectra during BSA incubated with glucose expressed by arbitrary fluorescence unit (AFU).**  $n = 4$ ,  $\bar{x} \pm s$ . <sup>a</sup> $P > 0.05$ , <sup>c</sup> $P < 0.01$  vs 0 wk.

| Incubation time/wk | Glucose/nmol · L <sup>-1</sup> |                         |                         |
|--------------------|--------------------------------|-------------------------|-------------------------|
|                    | 0                              | 50                      | 80                      |
| 0                  | 4.5 ± 0.9                      | 4.5 ± 0.9               | 4.5 ± 0.9               |
| 2                  | 4.6 ± 1.2                      | 4.2 ± 2.0 <sup>a</sup>  | 5.3 ± 2.7 <sup>a</sup>  |
| 4                  | 4.3 ± 1.3                      | 6.6 ± 1.4 <sup>c</sup>  | 8.2 ± 1.8 <sup>c</sup>  |
| 6                  | 4.4 ± 1.2                      | 14 ± 4 <sup>c</sup>     | 15.3 ± 2.9 <sup>c</sup> |
| 8                  | 4.3 ± 2.1                      | 15.2 ± 1.8 <sup>c</sup> | 19 ± 3 <sup>c</sup>     |
| 10                 | 4.7 ± 1.6                      | 17.2 ± 2.9 <sup>c</sup> | 23 ± 4 <sup>c</sup>     |
| 12                 | 4.4 ± 2.5                      | 24 ± 5 <sup>c</sup>     | 28 ± 5 <sup>c</sup>     |

### Effects of AGEPE-BSA on $[Ca^{2+}]_i$ of ASMC

The resting  $[Ca^{2+}]_i$  was  $121 \pm 4$  nmol · L<sup>-1</sup> in Hanks' solution containing  $Ca^{2+}$  1.3 mmol · L<sup>-1</sup>. AGEPE-BSA increased  $[Ca^{2+}]_i$  from  $121 \pm 4$  to  $492 \pm 20$  nmol · L<sup>-1</sup> in concentration- and time-dependent manners ( $P < 0.01$ ) (Tab 2).

After that time, the elevation of  $[Ca^{2+}]_i$ , slowly

**Tab 2. Effects of AGEPE-BSA on  $[Ca^{2+}]_i$  in ASMC.**  $n = 4$  wells,  $\bar{x} \pm s$ . <sup>b</sup> $P < 0.05$ , <sup>c</sup> $P < 0.01$  vs control.

| AGEPE-BSA  | $[Ca^{2+}]_i$ /nmol · L <sup>-1</sup> |                       |
|--|---------------------------------------|-----------------------|
| Concentration/mg · L <sup>-1</sup><br>(Incubation time = 40 min)             | 0 (Control)                           | 121 ± 4               |
|  | 25                                    | 185 ± 14 <sup>b</sup> |
|  | 50                                    | 227 ± 10 <sup>c</sup> |
|  | 100                                   | 352 ± 9 <sup>c</sup>  |
|  | 200                                   | 492 ± 20 <sup>c</sup> |
|  | 400                                   | 437 ± 16 <sup>c</sup> |
| Incubation time/min<br>(AGEPE-BSA concentration = 200 mg · L <sup>-1</sup> ) | 0 (Control)                           | 121 ± 4               |
|  | 10                                    | 206 ± 9 <sup>c</sup>  |
|  | 20                                    | 248 ± 14 <sup>c</sup> |
|  | 30                                    | 352 ± 19 <sup>c</sup> |
|  | 40                                    | 492 ± 20 <sup>c</sup> |
|  | 50                                    | 340 ± 46 <sup>c</sup> |
|  | 60                                    | 255 ± 12 <sup>c</sup> |

decreased, but still above the resting levels ( $P < 0.05$ ). The  $[Ca^{2+}]_i$  were also increased with the glucose concentration incubated with BSA and the time of glycosylation ( $P < 0.01$ ) (Tab 3).

**Tab 3. Effects of glycosylation and glucose modified BSA on  $[Ca^{2+}]_i$  in ASMC.**  $n = 6$  wells,  $\bar{x} \pm s$ . <sup>a</sup> $P > 0.05$ , <sup>b</sup> $P < 0.05$ , <sup>c</sup> $P < 0.01$  vs control.

|  | $[Ca^{2+}]_i$ /nmol · L <sup>-1</sup> |                       |
|--|---------------------------------------|-----------------------|
| Glycosylation time/wk                            | 0 (Control)                           | 121 ± 4               |
|  | 4                                     | 248 ± 14 <sup>b</sup> |
|  | 8                                     | 492 ± 20 <sup>c</sup> |
|  | 12                                    | 580 ± 37 <sup>c</sup> |
| Glucose concentration/<br>mmol · L <sup>-1</sup> | 0 (Control)                           | 121 ± 4               |
|  | 5                                     | 138 ± 12 <sup>a</sup> |
|  | 20                                    | 296 ± 16 <sup>c</sup> |
|  | 50                                    | 463 ± 10 <sup>c</sup> |
|  | 80                                    | 584 ± 19 <sup>c</sup> |

**Effects of AGEPE-BSA on DNA and protein synthesis in ASMC** AGEPE-BSA stimulated the incorporation of [<sup>3</sup>H]TdR and [<sup>3</sup>H]Leu into ASMC in concentration- and time-related manners ( $P < 0.01$ ), especially for [<sup>3</sup>H]TdR. The peak incorporation of [<sup>3</sup>H]TdR was 10 times compared with control ( $P < 0.01$ ). The incorporation of [<sup>3</sup>H]TdR and [<sup>3</sup>H]Leu were positively related with the time and glucose concentration of incubating BSA ( $P < 0.01$ ) (Tab 4).

## DISCUSSION

Our results showed that AGEPE-specific fluore-

**Tab 4. Effects of AGEP-BSA on the syntheses of DNA and protein in ASMC. *n* = 4 wells,  $\bar{x} \pm s$ . \**P* > 0.05, \*\**P* < 0.01 vs control.**

| AGEP-BSA                             |             | <sup>3</sup> H]Thymidine uptake, Bq/well | <sup>3</sup> H]Leucine uptake, Bq/well |
|--------------------------------------|-------------|--|--|
| Concentration/<br>mg·L <sup>-1</sup> | 0 (Control) | 12 ± 4                                   | 28 ± 5                                 |
|                                      | 50          | 31 ± 8 <sup>c</sup>                      | 30 ± 6 <sup>a</sup>                    |
|                                      | 100         | 68 ± 5 <sup>c</sup>                      | 72 ± 20 <sup>a</sup>                   |
|                                      | 200         | 111 ± 26 <sup>c</sup>                    | 89 ± 24 <sup>c</sup>                   |
|                                      | 300         | 86 ± 24 <sup>c</sup>                     | 92 ± 20 <sup>c</sup>                   |
| Incubated<br>time/min                | 0 (Control) | 12 ± 4                                   | 28 ± 5                                 |
|                                      | 6           | 39 ± 9 <sup>c</sup>                      | 33 ± 8 <sup>a</sup>                    |
|                                      | 12          | 103 ± 31 <sup>c</sup>                    | 52 ± 10 <sup>c</sup>                   |
|                                      | 24          | 209 ± 43 <sup>c</sup>                    | 68 ± 10 <sup>c</sup>                   |
|                                      | 36          | 254 ± 45 <sup>c</sup>                    | 162 ± 32 <sup>c</sup>                  |
| Glycosylation<br>time/wk             | 0 (Control) | 12 ± 4                                   | 28 ± 5                                 |
|                                      | 4           | 38 ± 5 <sup>c</sup>                      | 73 ± 21 <sup>c</sup>                   |
|                                      | 8           | 115 ± 30 <sup>c</sup>                    | 102 ± 29 <sup>c</sup>                  |
|                                      | 12          | 196 ± 26 <sup>c</sup>                    | 137 ± 34 <sup>c</sup>                  |

science increased with concentrations of glucose and lasting time of glycosylation, but due to limitation of time, we could not observe its dynamic changes more longer. Abnormal ASMC proliferation has a fundamental role in the pathogenesis of atherosclerosis. ASMC proliferation is regulated by many factors, such as hyperglycemia, insulin, hyperlipidemia, and some cytokines<sup>[4]</sup>. The results showed that the AGEP-BSA significantly stimulated the incorporation of [<sup>3</sup>H]TdR and [<sup>3</sup>H] Leu into ASMC in concentration-, interaction time-, glycosylating time-related manners, especially for [<sup>3</sup>H]TdR. The mechanisms by which AGEP stimulated incorporation of [<sup>3</sup>H]TdR and [<sup>3</sup>H]Leu into ASMC remain unclear. It is possible that when AGEP modified protein binds to its specific cell receptor, the production of some growth factors and cytokines are sufficient to stimulate the proliferation of ASMC.

It was found that abnormal incorporation of [<sup>3</sup>H]TdR and [<sup>3</sup>H]Leu into ASMC induced by AGEP-BSA was associated with elevation of [Ca<sup>2+</sup>]<sub>i</sub>. One mechanism may be its direct inhibitory effect on plasma membrane Ca<sup>2+</sup>-ATPase<sup>[9]</sup>, which could promote calcium inflow from the extracellular space. The second potential mechanism may involves AGEP-induced changes in signal transduction involved

diacylglycerol, IP<sub>3</sub>, and protein kinase C activity<sup>[8]</sup>.

The results may be of clinical significance. The stimulating effects of AGEP on incorporation of [<sup>3</sup>H]TdR and [<sup>3</sup>H]Leu into ASMC may underlie predisposition to atherogenesis and other chronic vascular complications among patients with diabetes. The target control of hyperglycemia and preventing the formation of AGEP by glycosylation inhibitor may contribute to reduce morbidity and mortality of diabetes and atherosclerosis.

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糖基化终产物对培养的大鼠主动脉平滑肌细胞增殖及胞浆游离钙含量的影响

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关键词 糖基化终产物; 钙; 血管平滑肌; 培养的细胞; 糖尿病血管病变; 动脉粥样硬化; 胸苷; 亮氨酸

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目的: 研究糖基化终产物(AGEP)对主动脉平滑肌细胞增殖的影响及其与 $[Ca^{2+}]_i$ 的关系. 方法: 采用同位素掺入法分别测定 DNA 和蛋白质合成; Fura 2-AM 测定 $[Ca^{2+}]_i$ . 结果: AGEP 以浓度、时间相关的方式促进 $[^3H]$ TdR 与 $[^3H]$ Leu 掺入细胞, 随 AGEP 作用时间、糖化时间延长, 掺入率

增加明显. AGEP 增加 $[Ca^{2+}]_i$ , 与时间、浓度相关, 但随 AGEP 作用时间延长(40 分钟后)而有所降低, BSA 修饰中葡萄糖浓度的增加, 糖基化时间延长,  $[Ca^{2+}]_i$  也呈上升趋势. 结论: AGEP 刺激平滑肌细胞增殖, 并与细胞 $[Ca^{2+}]_i$  浓度增加有关.

## Expression of receptor for advanced glycosylation end products (AGEP) and inhibition of AGEP-induced cytosolic calcium elevation by diltiazem in cultured rat aortic smooth muscle cells<sup>1</sup>

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**KEY WORDS** radioligand assay; advanced glycosylation end products; calcium; diltiazem; vascular smooth muscle; cultured cells; diabetic angiopathies; atherosclerosis; thoracic aorta

**AIM:** To study whether there is a high affinity receptor for advanced glycosylation end product (AGEP) on thoracic aorta smooth muscle cells (ASMC) and to test effect of diltiazem on elevation of cytosolic free calcium induced by AGEP.

**METHODS:** Interactions of AGEP-bovine serum albumin (BSA) with ASMC were studied with radioligand binding assay and cytosolic free calcium ( $[Ca^{2+}]_i$ ) was examined in cultured ASMC with Fura 2-AM. **RESULTS:** AGEP-BSA was specifically bound to cells at 4 °C and was taken up and degraded at 37 °C. These processes were concentration-dependent and saturable. Scatchard analysis indicated that the receptor was with dissociation constant of  $65.3 \pm 1.5 \text{ nmol} \cdot \text{L}^{-1}$  and its maximal binding capacity of  $1.57 \pm 0.04 \text{ nmol/g cell protein}$ . Early glycosylated low density lipoprotein (LDL) was not recognized by this receptor. AGEP-BSA elevated cytosolic free calcium in a concentration-dependent manner. Pretreatment

with diltiazem inhibited AGEP-BSA-induced elevation in concentration- and time-dependent manners.

**CONCLUSION:** There was a high affinity receptor for AGEP on ASMC, which mediated internalization and degradation of AGEP. Pretreatment with diltiazem inhibited the AGEP-induced elevation of cytosolic free calcium.

Advanced glycosylation end product (AGEP) resulted from the prolonged exposure of proteins to aldoses, such as glucose and ribose, as time and high glucose concentration function<sup>(1)</sup>. Accumulation of AGEP on long-lived proteins *in vivo* has been found to increase linearly with age and is accelerated in patients with diabetes. AGEP can form cross-links to and between proteins and interact with a class of binding sites on endothelial cell<sup>(2)</sup>, macrophage<sup>(3)</sup>, and mesangial cell<sup>(4)</sup>. Patients with diabetes are predisposed to atherosclerosis. AGEP may contribute to the pathogenesis of proliferative vascular lesion. Receptors for AGEP expressed on mesangial cell<sup>(4)</sup> and macrophage<sup>(5)</sup> play a potential role in removal of senescent macromolecules and tissue remodeling. The discovery of this receptor on vasculature<sup>(6)</sup> reasonably link the progressive accumulation of AGEP in patient with diabetes and vascular complication. Expression of receptor for AGEP was increased in endothelial cells in patients with diabetes or extensive peripheral

<sup>1</sup> Project supported by the National Natural Science Foundation of China, No 39470314.

<sup>2</sup> Correspondence to Prof XIE Pei-Li. Phn: 86-25-330-1508, ext 2141. Received 1996-11-18 Accepted 1997-06-04