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.

BIBLID: ISSN 0253-9756 Acta Pharmacologica Sinica 中国药理学报 1997 Sep; 18 (5): 425 - 430

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[1]

ZHOU Qiu-Gen, LIU Nai-Feng, XIE Pei-Li[2] (*Department of Cardiology, The First Affiliated Hospital, Nanjing Railway Medical College, Nanjing 210009, China*)

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 ℃ and was taken up and degraded at 37 ℃. These processes were concentration-dependent and saturable. Scatchard analysis indicated that the receptor was with dissociation constant of 65.3 $\pm$1.5 nmol·L^{-1} and its maximal binding capacity of 1.57$\pm$0.04 nmol/g cell protein. Early glycated 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[1]. 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[2], macrophage[3], and mesangial cell[4]. Patients with diabetes are predisposed to atherosclerosis. AGEP may contribute to the pathogenesis of proliferative vascular lesion. Receptors for AGEP expressed on mesangial cell[4] and macrophage[5] play a potential role in removal of senescent macromolecules and tissue remodeling. The discovery of this receptor on vasculature[6] 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

[1] Project supported by the National Natural Science Foundation of China, No 39470314.

[2] Correspondence to Prof XIE Pei-Li. Phn: 86-25-330-1508, ext 2141.

Received 1996-11-18 Accepted 1997-06-04

atherosclerotic vascular disease[7]. We proposed that AGEP might interact with aortic smooth muscle cells (ASMC) and modulate their cellular functions. In present study, the interactions of ASMC with AGEP-bovine serum albumin (BSA) were characterized and the effect of diltiazem on AGEP-BSA-induced elevation $[Ca^{2+}]_i$ was tested.

MATERIALS AND METHODS

Culture of smooth muscle cells ASMC was cultured with thoracic aorta excised from Sprague-Dawley rats by substrate-attached explants and incubated in Dulbecco's modified Eagle's medium (DMEM). ASMC was identified by typical growth pattern of "hills and valleys" under the phase-contrast microscope. Cells available for experiments were 6 – 18 passages and were counted and seeded in the plastic 24 wells dish.

Preparation and radiolabeling of AGEP-BSA AGEP-BSA was prepared by incubating BSA in PBS buffer 10 mmol·L^{-1}, pH 7.4, with glucose 50 mmol·L^{-1} at 37 ℃ for 6 wk in the presence of phenylmethylsulfonyl fluoride 1.5 mmol·L^{-1}, edetic acid 0.5 mmol·L^{-1} and antibiotics (benzylpenicillin 10^5 U·L^{-1} and gentamicin 40 mg·L^{-1}) as described previously[2]. AGEP-BSA exhibited characteristic yellow-brown pigment and fluorescence. Aliquots of AGEP-BSA were dialyzed extensively against PBS. Radiolabeling of AGEP-BSA was accomplished by the chloramine-T method with carrier [^{125}I]-NaI. Samples were dialyzed against a 1000-fold excess PBS 10 mmol·L^{-1} pH 7.4 until at least 95 % of the radioactivity was trichloroacetic acid precipitable and the samples were iodide free. Protein concentrations were determined[8]. Specific activity of labeled AGEP-BSA was 16.67 GBq·g^{-1}.

Preparation of early glycated low density lipoprotein (LDL) Human LDL was isolated from health volunteer. Early glycated LDL was prepared by aseptically incubating LDL (2 g·L^{-1}) in buffer PBS 10 mmol·L^{-1}, pH 7.4, with glucose 80 mmol·L^{-1} at 37 ℃ for 1 wk in the presence of edetic acid 1 mmol·L^{-1}. Before experiments, the sample were dialyzed against PBS.

Radioligand binding and internalization and degradation analysis These studies were performed as described[3] with some modifications. All experiments were performed in triplicate.

Binding experiments were carried out at 4 ℃. After 12-h seeded in 24 wells linbro plastic dishes, $1.5 \times 10^6 - 2.0 \times 10^6$ cells in each well were washed 3 times and allowed to equilibrate at 4 ℃ in fresh DMEM. Binding was initiated by aspirating the medium and adding DMEM 1.0 mL in each well, containing labeled AGEP-BSA 10, 50, 100, 150, 300, or 350 nmol·L^{-1}. Cells were incubated at 4 ℃ for 4 h. The radioligand-containing medium then was aspirated and cells were gently washed 3 times with ice-cold Hanks' solution. Cells per well were dissolved for 12 h in 1.0 mL NaOH 0.1 mol·L^{-1}. Cell-associated radioactivity was determined with γ counter (SN-682B). Total binding was defined as the amount of labeled AGEP-BSA bound. Nonspecific binding was defined as the radioligand bound in the presence of an 100-fold of unlabeled AGEP-BSA (1, 5, 10, 15, 20, 25, 30, 35 μmol·L^{-1}). The difference between total and nonspecific binding was specific binding. Transformation of saturation binding data was performed according to Scatchard to estimate the maximal binding capacity and dissociation constant.

For the following experiments at 37 ℃, cell-associated radioactivity was divided into 2 pools: membrane-bound radioligand, eluted by 2 min exposure of cells to buffer containing heparin/edetic acid solution, and internalized radioligand. ASMC internalization experiments were performed at 37 ℃. Attached cells (2×10^6) in each well were incubated with ^{125}I-AGEP-BSA 75, 150, 225, 300, 375, or 600 nmol·L^{-1} for 4 h. The cell monolayer then was gently washed 3 times with ice-cold Hanks' solution to remove extracellular radioligand and then was washed with heparin/edetic acid solution to remove membrane-bound radioligand. Cells were lysed by addition of NaOH 0.1 mol·L^{-1}. Cell-associated radioactivity representing intracellular accumulation of ^{125}I-labeled AGEP-BSA was measured. Competition of internalization was studied under the same condition in the presence of ^{125}I-AGEP-BSA 150 nmol·L^{-1} in each well, added glycated LDL 0.75, 1.5, 3, 4.5, 12 μmol·L^{-1}.

Degradation experiment was done as follows: Attached cells (2×10^6) in each well were incubated with ^{125}I-labeled AGEP-BSA 150 nmol·L^{-1} at 4 ℃ for 4 h. Cells then were warmed at 37 ℃ to continue incubating. The cell-associated radioactivity representing intracellular accumulation of radioligand and trichloroacetic acid soluble material radioactivity representing metabolite of radioligand in the medium in each well during 1, 2, 3, 4, and 5 h were determined.

The results were expressed as kBq/g cell protein.

Measurement of cytosolic Ca^{2+} The experiments were carried out as described[9] with modifications. Briefly, deattached cells (2×10^6 cells/well) were incubated in DMEM containing Fura-2 acetoxymethyl ester dissolved in Me_2SO 5 μmol·L^{-1} and BSA 0.1 % at 37 ℃ in a dark compartment for 40 min. Cells then were washed 3 times with buffer medium (NaCl 145, KCl 5, $MgCl_2$ 1, glucose 5, $CaCl_2$ 1, NaH_2PO_4 0.5, HEPES 10 mmol·L^{-1}, at pH 7.4) by centrifugation at 50 × g for 5 min and resuspended. Loaded cells were available for $[Ca^{2+}]_i$ measurement at 20 – 25 ℃ in 2-h. Fluorescence was measured using F-3000 spectrofluorometer (Hitachi, Japan) at λ_{ex} 340 nm and λ_{em} 500 nm. Autofluorescence was measured with unloaded cells. $[Ca^{2+}]_i = K_D \cdot (F - F_{min})/(F_{max} - F)$. The F_{max} and F_{min} were determined by Triton-100 and egtazic acid, respectively.

Agents Fura-2 acetoxymethyl ester (Sigma); Dulbecco's modified Eagle's medium (Gibco); Diltiazem (Sigma); [^{125}I]NaI (with carrier, China Institute of Atomic Energy); Sprague-Dawley rats (n = 3 wt 180 – 200 g, ♂ ♀, Clean animal, Animal Center of Railway Department).

Statistic analysis Data were expressed as $\bar{x} \pm s$ and compared by t-test and ANOVA.

RESULTS

Binding, internalization, degradation of AGEP-BSA by ASMC When ASMC were incubated at 4 ℃ with increasing concentrations of ^{125}I-AGEP-BSA, specific binding increased in a saturable fashion (Fig 1A). Scatchard analysis (Fig 1B) indicated that the high affinity receptor for AGEP-BSA was with a dissociation constant of 65.3 ± 1.5 nmol · L^{-1} and maximal specific binding of 1.57 ± 0.04 nmol/g cell protein.

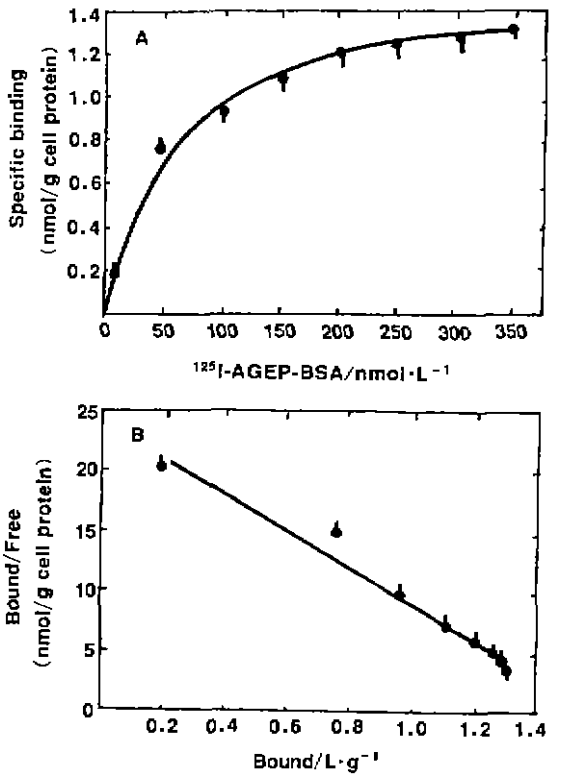

Fig 1. Specific binding of ^{125}I-AGEP-BSA to cultured rat ASMC and Scatchard analysis. n = 3, $\bar{x} \pm s$.

When ASMC were incubated at 37 ℃ for 4 h with increasing concentrations of ^{125}I-labeled AGEP-BSA, intracellular accumulation of radioligand increased in a saturable fashion (Fig 2A). AGEP-BSA effectively competed with labeled AGEP-BSA, suppressing internalization to about 8 % of maximal accumulation. The early glycated LDL did not competed with labeled AGEP-BSA (Fig 2B).

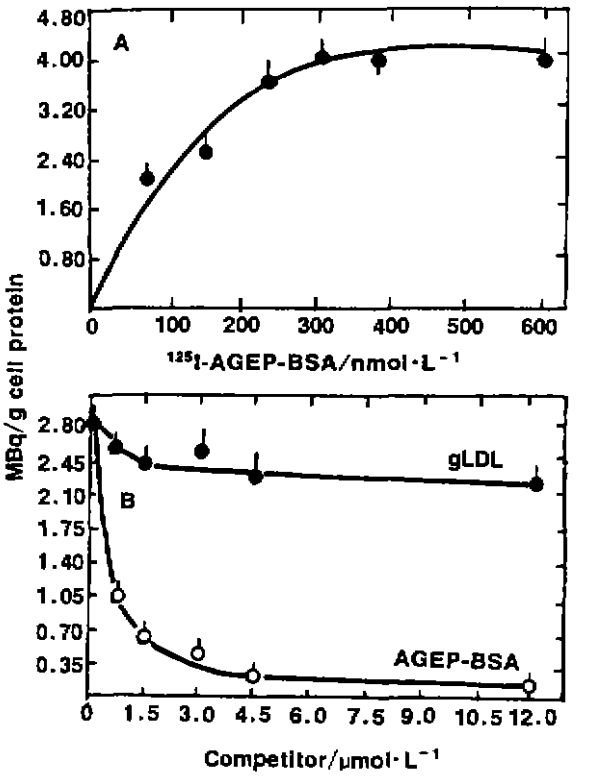

Fig 2. (A) Intracellular accumulation of ^{125}I-AGEP-BSA by cultured rat ASMC. (n = 3 wells, $\bar{x} \pm s$); (B) Internalization of ^{125}I-AGEP-BSA by ASMC in the presence of unlabeled competitors. The intracellular radioactivity in each well containing ^{125}I-AGEP-BSA 150 nmol · L^{-1} and the indicated concentrations of AGEP-BSA or gLDL were measured after 4 h incubation. n = 3 wells, $\bar{x} \pm s$.

When cells were incubated with ^{125}I-labeled AGEP-BSA (150 nmol · L^{-1}) at 4 ℃ for 4 h and warmed at 37 ℃ to continue incubating, Cell-associated radioactivity declined, while trichloroacetic acid soluble radioactivity in the medium increased concomitantly (Fig 3).

AGEP-BSA-induced [Ca^{2+}]$_i$ elevation The resting [Ca^{2+}]$_i$ was 110 ± 10 nmol · L^{-1} in buffer medium containing Ca^{2+} 1.0 mmol · L^{-1}. AGEP-BSA

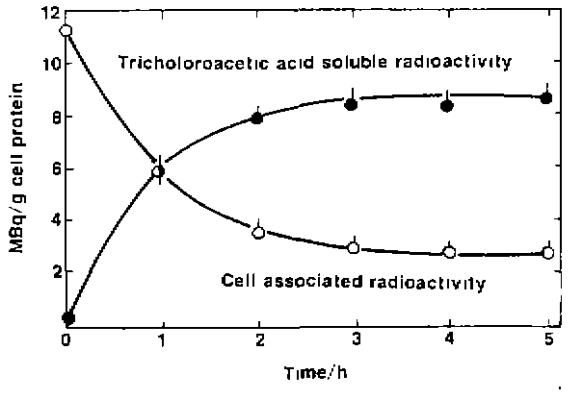

Fig 3. Degradation at 37 ℃ of ^{125}I-AGEP-BSA previously bound at 4 ℃ by ASMC. Cells incubated with ^{125}I-AGEP-BSA 150 nmol·L^{-1} at 4 ℃ for 4 h were then warmed at 37 ℃. At indicated interval, cell associated radioactivity and trichloroacetic acid soluble radioactivity in the medium were determined in triplicate samples. $n = 3$ wells, $\bar{x} \pm s$.

increased the $[Ca^{2+}]_i$ in a concentration dependent manner, from 110 ± 10 nmol·L^{-1} to 150 ± 10, 200 ± 6, 241 ± 9, 269 ± 18, 321 ± 20 nmol·L^{-1} at AGEP-BSA 30, 75, 150, 200, 300 nmol·L^{-1}, respectively (Tab 1).

Tab 1. Effect of AGEP-BSA on cytosolic free calcium in rat cultured ASMC. ($n = 3$ wells, $\bar{x} \pm s$. $^cP < 0.01$ *vs* control, $F = 66.22$).

AGEP-BSA/nmol·L^{-1}	$[Ca^{2+}]_i$/nmol·L^{-1}
0	110 ± 10
30	150 ± 10^c
75	200 ± 6^c
150	241 ± 9^c
200	269 ± 18^c
300	321 ± 20^c

Diltiazem on AGEP-BSA-induced $[Ca^{2+}]_i$ elevation Pretreatment with diltiazem 50 μmol·L^{-1} for 0, 10, 20, 30, 40 min inhibited the AGEP-BSA-induced $[Ca^{2+}]_i$ elevation, respectively. After ASMC were stimulated by AGEP-BSA for 10 min, diltiazem had no effect on AGEP-BSA-induced $[Ca^{2+}]_i$ elevation (Tab 2). Pretreatment with diltiazem for 20 min inhibited the AGEP-BSA-induced $[Ca^{2+}]_i$ elevation in a concentration-dependent manner, from 321 ± 20 nmol·L^{-1} to 284 ± 9, 246 ± 13, 222 ± 7, 185 ± 7, 152 ± 9 nmol·L^{-1} at 10, 25, 40, 50, 60 μmol·L^{-1}, respectively (Tab 3).

Tab 2. Time-dependent effect of diltiazem (50 μmol·L^{-1}) on AGEP-BSA-induced $[Ca^{2+}]_i$ elevation in ASMC. ($n = 3$ wells, $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control, $F = 51.30$) (negative mark of time indicated pretreatment with diltiazem before stimulated by AGEP-BSA; zero meaned diltiazem and AGEP-BSA were added concomitantly).

Time of treatment/min	$[Ca^{2+}]_i$/nmol·L^{-1}
Control	321 ± 20
−40	170 ± 8^c
−30	175 ± 6^c
−20	185 ± 5^c
−10	203 ± 7^c
0	276 ± 7^b
10	322 ± 6^a

Tab 3. Concentration-dependent effect of pretreatment for 20 min with diltiazem on AGEP-BSA-induced $[Ca^{2+}]_i$ elevation in ASMC. ($n = 3$ wells, $\bar{x} \pm s$. $^cP < 0.01$ *vs* control, $F = 79.88$).

Diltiazem/μmol·L^{-1}	$[Ca^{2+}]_i$/nmol·L^{-1}
Control	321 ± 20
10	284 ± 9^c
25	246 ± 13^c
40	222 ± 7^c
50	185 ± 7^c
60	159 ± 9^c

DISCUSSION

In this work, it has been demonstrated that there is a specific high affinity receptor for AGEP-BSA on ASMC. That intracellular accumulation of radioligand increases in a saturable fashion and is inhibited by unlabeled AGEP-BSA show that this process is receptor-mediated. Meanwhile, appearance of trichloroacetic acid soluble material shows that internalized radioligand will gradually degraded. These facts suggest that AGEP-BSA is taken up and degraded by ASMC through this kind of receptor. Some experts discovered that early glycated LDL induced oxidant stress on endothelial cells[10]. Our result showed that early glycated LDL was not taken up by this kind of receptor. The mechanism that glycated LDL exerts its cellular effect remains further investigation. In this work, it is also showed that receptors for AGEP on ASMC have similar dissociation

constant with that on macrophage. This receptor expressed on ASMC may play some role in removal of excess accumulation of AGEP in patients with diabetes and contribute to atherogenesis. The identification and isolation of two AGEP binding proteins on animal and human macrophage and vascular endothelial cell further support the concept that the receptor for AGEP plays a central role in specific effects of AGEP on target cells[11]. But the physiological significance of the receptor for AGEP on ASMC remains elucidated. Further investigations are needed to isolate and analyze the receptor on ASMC.

AGEP-BSA induced $[Ca^{2+}]_i$ elevation. AGEP interacting with their endothelial receptor could induce expression of vascular cell adhesion molecule-1[12], which has been associated with early stage of atherosclerosis, and their interaction could also induce increased vascular permeability[13], which is characteristic of diabetic vasculopathy. Overload of $[Ca^{2+}]_i$ is involved in atherosclerosis. It is reasonable to propose that AGEP-induced $[Ca^{2+}]_i$ elevation in ASMC at least partially contribute to patients with diabetes being predisposed to atherosclerosis. Pretreatment with diltiazem significantly, but not completely, inhibited AGEP-induced $[Ca^{2+}]_i$ elevation, suggesting that calcium channel is involved in AGEP-induced $[Ca^{2+}]_i$ elevation. How calcium channel is stimulated remains unclear. Intracellular mechanisms involved in AGEP-induced $[Ca^{2+}]_i$ elevation are being investigated. But the effect of diltiazem shows a new light into prevention of diabetic vascular complication. It is much better to bear the mind that this is *in vitro* information. Further *in vivo* studies remain to be performed.

Our observations suggest that there is a specific high affinity receptor for AGEP-BSA on ASMC which not only mediates internalization and degradation of AGEP-BSA, but also mediates AGEP-BSA-induced $[Ca^{2+}]_i$ elevation. Diltiazem significantly, not completely, inhibits AGEP-BSA-induced $[Ca^{2+}]_i$ elevation.

REFERENCES

1 Brownlee M. Advanced protein glycosylation in diabetes and aging. Annu Rev Med 1995; 46: 223 – 34.

2 Esposito C, Gerlach H, Brett J, Stern D, Vlassara H Endothelial receptor-mediated binding of glucose-modified albumin is associated with increased monolayer permeability and modulation of cell surface coagulant properties. J Exp Med 1989; 170: 1387 – 407.

3 Vlassara H, Brownlee M, Cerami A. High-affinity-receptor-mediated uptake and degradation of glucose-modified proteins: a potential mechanism for the removal of senescent macromolecules. Proc Natl Acad Sci USA 1985; 82: 5588 – 92.

4 Skolnik EY, Yang Z, Makita Z, Radoff S, Kirstein M, Vlassara H Human and rat mesangial cell receptors for glucose-modified proteins: potential role in kidney tissue remodeling and diabetic nephropathy. J Exp Med 1991; 174: 931 – 9.

5 Vlassara H, Brownlee M, Manogue KR, Dinarello CA, Pasagian A. Cachectin/TNF and IL-1 induced by glucose-modified proteins: role in normal tissue remodeling. Science 1988; 240: 1546 – 8.

6 Schmidt AM, Vianna M, Gerlach M, Brett J, Ryan J, Kao J, *et al* Isolation and characterization of two binding proteins for advanced glycosylation end products from bovine lung which are present on the endothelial cell surface. J Biol Chem 1992; 267: 14987 – 97

7 Ritthaler U, Deng Y, Zhang Y, Greten J, Abel M, Sido B, *et al*. Expression of receptors for advanced glycation end products in peripheral occlusive vascular disease. Am J Pathol 1995; 146: 688 – 94.

8 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951; 193: 265 – 75

9 Malgaroli A, Milani D, Meldolesi J, Pozzan T. Fura-2 measurement of cytosolic free Ca^{2+} in monolayers and suspensions of various types of animal cells. J Cell Biol 1987; 105: 2145 – 55.

10 Mullarkey CJ, Edelstein D, Brownlee M. Free radical generation by early glycation products: a mechanism for accelerated atherogenesis in diabetes. Biochem Biophys Res Commun 1990; 173: 932 – 9.

11 Schmidt AM, Hasu M, Popov D, Zhang JH, Chen JX, Yan SD, *et al*. Receptor for advanced glycation end products (AGEs) has a central role in vessel wall interactions and gene activation in response to circulating AGEP proteins Proc Natl Acad Sci USA 1994; 91: 8807 – 11.

12 Schmidt AM, Hori O, Chen JX, Li JF, Crandall J, Zhang JH, *et al*. Advanced glycation end products interacting with their endothelial receptor induce expression of vascular cell adhesion molecule-1 (VCAM-1) in cultured human endothelial cells and in mice. J Clin Invest 1995; 96: 1395 – 403.

13 Wautier JL, Zoukourian C, Chappey O, Wautier MP, Guillausseau PJ, Cao R, *et al*. Receptor-mediated endothelial cell dysfunction in diabetic vasculopathy. soluble receptor for advanced glycation end products blocks hyperpermeability in diabetic rats. J Clin Invest 1996; 97: 238 – 43.

425-430

培养的大鼠主动脉平滑肌细胞表达糖基化终产物受体和地尔硫䓬对糖基化终产物升高胞浆游离钙的抑制

周秋根, 刘乃丰, 谢陪俐 (南京铁道医学院第一附属

医院心内科，南京 210009，中国）

关键词 放射配基测定；糖基化终产物；钙；地尔硫䓬；血管平滑肌；培养的细胞；糖尿病血管病变；动脉粥样硬化；胸主动脉

目的：探讨培养的主动脉平滑肌细胞(ASMC)上是否表达糖基化终产物(AGEP)高亲性受体和地尔硫䓬对 AGEP 升高胞浆游离钙的抑制．**方法**：用放射配基结合方法研究 ASMC 与 AGEP 的相互作用；用钙离子荧光指示剂 Fura 2-AM 测定 ASMC 胞浆游离钙．**结果**：ASMC 上有 AGEP 高亲和性受体表达，其解离常数为 65.3 ± 1.5 nmol·L^{-1}，最大结合容量 1.57 ± 0.04 nmol/g 细胞蛋白；通过该受体介导、平滑肌细胞可内化、代谢 AGEP．AGEP 使胞浆游离钙呈浓度依赖性升高；地尔硫䓬呈时间、浓度依赖性抑制此效应．**结论**：大鼠 ASMC 上有 AGEP 高亲和性受体表达；地尔硫䓬可抑制 AGEP 介导的胞浆游离钙升高．