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Effect of advanced glycosylation end products on diacylglycerol signaling pathway in cultured rat aortic smooth muscle cells[1]

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KEY WORDS advanced glycosylation end products; diglycerides; vascular smooth muscle; cultured cells; atherosclerosis; signal transduction; vitamin E; guanidines; phosphatidic acids; autoradiography

ABSTRACT

AIM: To investigate whether diacylglycerol (Dia) signaling pathway is stimulated by advanced glycosylation end products (AGEP) and to determine effect of vitamin E and aminoguanidine on Dia level induced by AGEP in cultured rat aortic vascular smooth muscle cells (VSMC). **METHODS**: The effect of AGEP on Dia level in cultured VSMC was measured by radio-enzymatic assay. Quantitative measurements of [^{32}P]phosphatidic acid were performed by thin-layer chromatography and autoradiography. **RESULTS**: The Dia level in VSMC incubated with AGEP-BSA was markedly increased in a concentration-dependent, biphasic manner. The early phase was rapid and transient, peaking at 15 s. The late phase reached the maximal level at 10 min and decayed slowly. The Dia levels in VSMC exposed to different concentrations of AGEP-BSA and AGEP-BSA glycosylated for various periods were greatly increased compared with control group. Vitamin E 50, 100 nmol · L^{-1} prevented the AGEP-BSA-stimulated elevation of Dia level in VSMC, from (940 ± 43) pmol · L^{-1} to (599 ± 38), (290 ± 21) pmol · L^{-1}, respectively. In aminoguanidine-treated AGEP-BSA groups, Dia level in the cells incubated with the same concentration of AGEP-BSA (100, 200 mg · L^{-1}) were decreased to (260 ± 8) and (289 ± 10) pmol · L^{-1}, respectively. Early glycosylated low-density lipoproteins (LDL) did not affect Dia level. **CONCLUSION**: AGEP causes a robust stimulation of Dia signaling pathway in VSMC. Vitamin E and aminoguanidine attenuate the production of Dia.

INTRODUCTION

Advanced glycosylation end products (AGEP) are formed by the interaction of an aldose with NH_2 of proteins, and the subsequent Amadori rearrangement leads to complex molecules. The accelerated formation of AGEP is observed in conditions such as diabetes mellitus and aging, renal insufficiency, which are closely associated with vascular diseases[1]. Therefore, AGEP may play a crucial role in aging processes, development of diabetic complications, and atherosclerosis. Accumulation of intracellular AGEP has been found in vascular smooth muscle cells (VSMC)-derived foam cells in vascular fatty streak. Significant extracellular accumulation of AGEP was also observed in advanced lesions of atherosclerotic plaque[2]. Recent studies have found that AGEP mediates the chemotaxis of rabbit VSMC[3]. Pathogenic properties of AGEP were mainly mediated by interaction with its specific receptors. Our group previously reported that there was a specific high affinity receptor for AGEP-BSA on VSMC[4], and AGEP-BSA remarkably stimulated the proliferation of VSMC and increased intracellular $[Ca^{2+}]_i$[5]. The related changes of signal transduction after AGEP binding to its receptors were considered as key components of pathogenic properties of AGEP, but defined mechanism is not fully understood. Since diacylglycerol (Dia) activates protein kinase C that appears to be a key enzyme for many cellular responses, we proposed that one potential mechanism might be responsible for AGEP-induced

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signal transduction, which is involved with changes of Dia level. This study was designed to investigate the effects of AGEP on Dia level and pharmacological intervention in cultured VSMC.

MATERIALS AND METHODS

Cell culture VSMC was obtained from thoracic and descending aorta excised from Spague-Dawley rats (Certificate № 97002) and isolated by substrate-attached explants outgrowing. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal bovine serum (FBS), supplemented with *L*-glutamine 2 mmol, benzylpenicillin 100 U at 37 ℃, 5 % CO_2. VSMC was identified by typical growth pattern of 'hills and valleys' under the phase-contrast microscope, the cells available for experiments were 4 – 6 passages and seeded in the plastic 6-well plates. After reaching confluence, the cells were passaged and subcultured into 6-well and 24 well plastic dishes with a split ration of 1:2.

Preparation of AGEP-BSA AGEP-BSA was prepared by incubating bovine serum albumin (BSA) in phosphate buffer solution (PBS) 10 $mmol \cdot L^{-1}$, pH 7.4, with glucose 50 $mmol \cdot L^{-1}$ at 37 ℃ for indicated periods of time in the presence of phenylmethylsulfonyl fluoride 1.5 $mmol \cdot L^{-1}$, edetic acid 0.5 $mmol \cdot L^{-1}$, and antibiotics (benzylpenicillin 100 $kU \cdot L^{-1}$ and gentamicin 40 $mg \cdot L^{-1}$). Aminoguanidine (AG) 100 $mmol \cdot L^{-1}$ was added into one of AGEP-BSA groups, and incubated at 37 ℃ for 4 – 8 wk. AGEP-BSA exhibited characteristic yellow-brown pigment and fluorescence (at 450 nm on excitation, at 390 nm at a fluorescence spectrometor). Aliquots of AGEP-BSA were dialyzed extensively against PBS before experiments.

Isolation and glycosylation of low-density lipoprotein (LDL) Human serum LDL was isolated by ultracentrifugation. Early glycated LDL was prepared by aseptically incubating LDL (2.0 $g \cdot L^{-1}$) in PBS (10 $mmol \cdot L^{-1}$, pH 7.4) with glucose 80 $mmol \cdot L^{-1}$ at 37 ℃ for 1 wk in the presence of edetic acid 1.0 $mmol \cdot L^{-1}$. Before experiments, the samples were dialyzed against PBS.

Quantitative measurement of intracellular Dia The cells were seeded in a 6-well plate at a density of $4 \times 10^6 - 5 \times 10^6$ cells/well in DMEM containing 10 % FBS and allowed to attach overnight. The cells were washed twice with DMEM and incubated with serum-free DMEM for 24 h. After washed twice with DMEM again, the cells were incubated with AGEP-BSA at 37 ℃ for the indicated periods of time and concentrations. The incubation was terminated by aspiration of culture medium and immediate addition of 1.0 mL cold methanol. The cells were scraped into methanol and added to 1.0 mL chloroform. The plates were washed with an additional 1.0 mL cold methanol, which was also added to the chloroform. The lipids were then extracted by the method described[5], except that NaCl 1.0 $mmol \cdot L^{-1}$ was used instead of distilled water. Following centrifugation at $5000 \times g$ for 2 min, lower chloroform phase was analyzed for Dia within 72 h as described[6]. The samples were pipetted into tubes, then were evaporated and solubilized. Each sample was added with sample-reaction buffer containing *Escherichia coli* Dia kinase, [γ-^{32}P]ATP solution. The samples were streaked onto silica gel thin-layer plates in a running solvent of chloroform : methanol : acetic acid. The radioactive spot corresponding to PA was visualized by auto-radiography, scraped, and quantified by liquid scintillation counting (Beckman LS 5000TD). According to manufacture's instruction of the Dia assay Kit, the results were calculated from standard curve and expressed as $pmol \cdot L^{-1}$.

Reagents Dulbecco's modified Eagle's medium (DMEM, Gibco); Dia kit (Amersham); [γ-^{32}P]ATP (with carrier, China Institute of Atomic Energy); Sprague-Dawley rats ($n = 5$, wt 150 – 180 g, male, grade Ⅱ, Animal Center of Nanjing Railway Medical College). Scintillation liquid was xylene containing diphenyloxazole 4.0 $g \cdot L^{-1}$; PPO and 1,4-di-[2-(5-phenyloxazolyl)]-benzene 0.1 $g \cdot L^{-1}$, POPOP. All other reagents were of AR.

Statistical analysis All numerical data were expressed as $\bar{x} \pm s$. Statistical analysis was performed by program "Statistic Graphic" and variance analysis (ANVOA) was applied.

RESULTS

AGEP-induced Dia accumulation When the cells were treated with AGEP-BSA 100 $mg \cdot L^{-1}$ for different period, the Dia level exhibited biphasic, with

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a peak at 15 s and a second peak at 10 min. The Dia level was consistently sustained above baseline for at least 10 min (Fig 1).

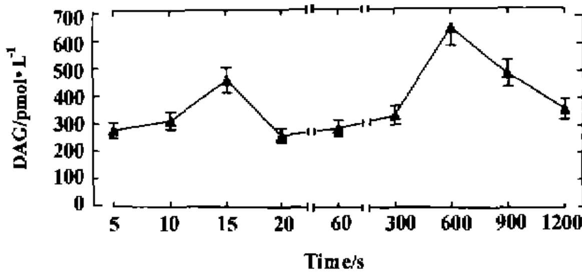

Fig 1. Dia formation by AGEP-BSA in VSMC. A biphasic increase of Dia in cultured VSMC induced by AGEP-BSA. $n=3$. $\bar{x} \pm s$.

When the cells were incubated with AGEP-BSA (50, 100, 200 mg·L^{-1}) at 37 ℃ for 10 min, AGEP increased Dia level from (328 ± 15) to (940 ± 43) pmol · L^{-1} in concentration-dependent manners (Fig 2).

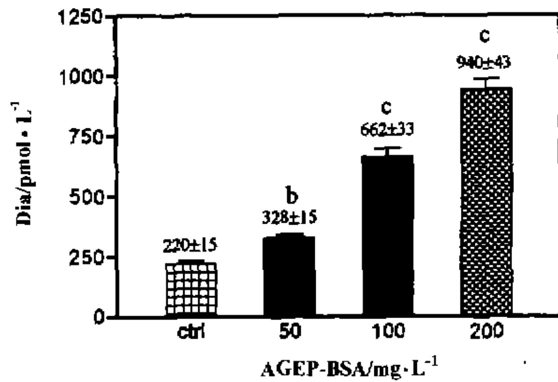

Fig 2. Concentration-response curves of Dia production in VSMC by AGEP-BSA. $n=3$. $\bar{x} \pm s$. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control.

When VSMC were treated with different AGEP-BSA samples which were glycosylated for different time at the same concentration (200 mg·L^{-1}), the Dia level was also increased with the time of glycosylation (Fig 3).

When the cells were incubated with AGEP-BSA which was glycosylated with different concentrations of glucose, the Dia levels were increased related to the concentrations of glucose (Fig 4).

Effect of gLDL on Dia levels in VSMC

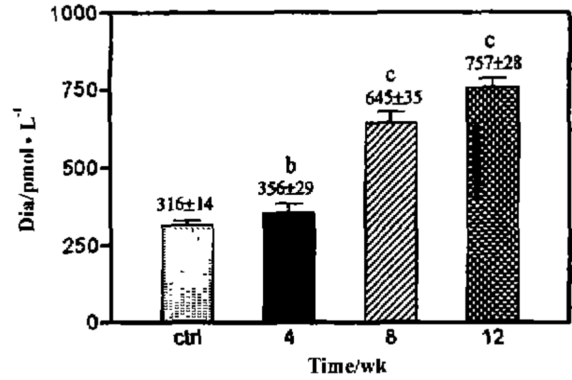

Fig 3. Effect of AGEP-BSA modified for different time on Dia level in VSMC. $n=3$. $\bar{x} \pm s$. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control.

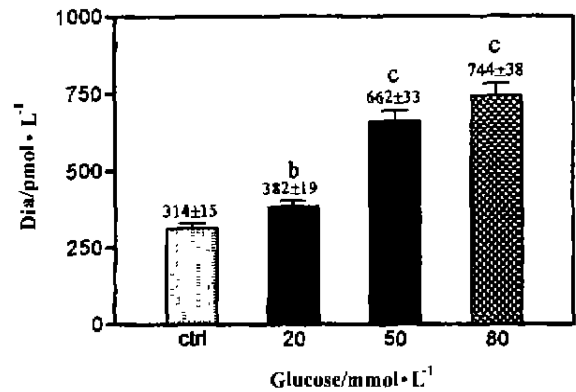

Fig 4. Effects of AGEP-BSA glycosylated by different concentrations of glucose (8 wk) on Dia level in VSMC. $n=3$. $\bar{x} \pm s$. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control.

The cells were treated with 50, 100 mg·L^{-1} of gLDL at 37 ℃ for 10 min, the Dia levels were 264, 262 pmol·L^{-1} respectively, compared with that of control group (267 pmol·L^{-1}).

Effect of vitamine E on Dia levels in VSMC Vitamin E 50, 100 nmol·L^{-1} inhibited the production of Dia induced by AGEP-BSA (Fig 5).

Effect of AG on Dia levels in VSMC The cells were treated with the same concentration of AGEP-BSA (100, 200 mg·L^{-1}) for 10 min, which was incubated with AG 100 mmol·L^{-1} at 37 ℃ for 8 wk, Dia level in these cells were decreased (Fig 6).

Effect of AG on arbitrary fluorescence unit (AFU) of AGEP-BSA BSA incubated with glucose and without glucose for 8 wk presented (29 ± 5),

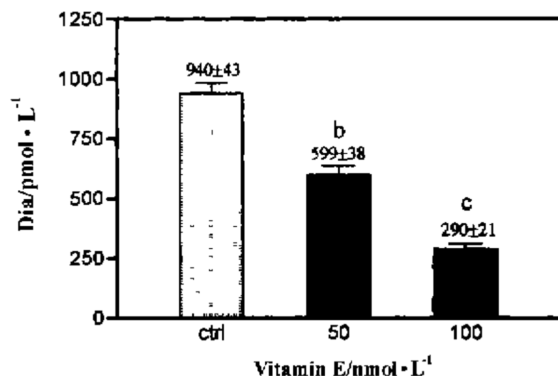

Fig 5. Effect of vitamin E on Dia level in VSMC stimulated by AGEP-BSA. $n=3$. $\bar{x}\pm s$. $^bP<0.05$, $^cP<0.01$ *vs* control.

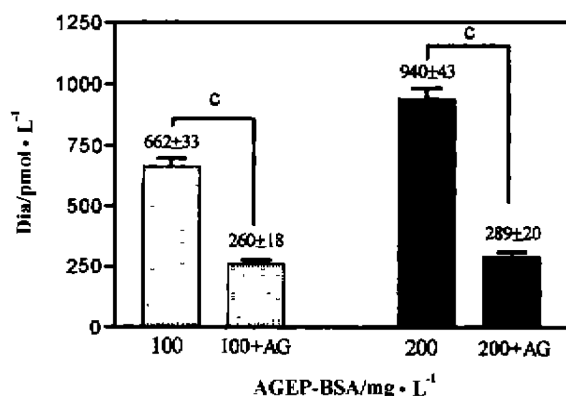

Fig 6. Effects of AGEP-BSA treated with AG on Dia level in VSMC. $n=3$. $\bar{x}\pm s$. $^cP<0.01$ *vs* the group without AG.

(8 ± 2) AFU, respectively. When BSA was incubated with glucose and AG ($10\ \text{mmol}\cdot\text{L}^{-1}$) for the same time, AG markedly ($P<0.05$), but not completely attenuated production of AFU of AGEP-BSA to (14 ± 2) AFU.

DISCUSSION

The production of Dia is related to two intracellular pathways. One is *de novo* synthesis of Dia from glucose, the other is receptor-mediated hydrolysis of membrane phospholipids via G protein which can activate phospholipase C (PLC). Our results showed that AGEP-BSA induced a biphasic elevation of Dia level. Such a transient and sustained Dia accumulation could presumably occur from phospholipase C-mediated hydrolysis of a major membrane phospholipid such as phosphatidylinositol (PI) or phosphatidylcholine (PC). Similarly, angiotensin II and endothelin also stimulate a biphasic production of Dia[7]. Although the hydrolysis of PI by PLC is a well-described intracellular source of Dia, recent studies demonstrated that PC was also a source for production of Dia in VSMC. It should be noted that the cells contain considerably more PC than PI, therefore, more Dia can be generated from the hydrolysis of PC. Dia generated from the hydrolysis of PI activates PKC, which down-regulates PI-PLC activity. This limits the amount of Dia generated in the cell through this pathway[8]. It was found that multiple Dia species were derived from several sources and different Dia species might be differentially metabolized[9]. This suggests that, in addition to the differential production of the various Dia moieties, some species slowly metabolized might contribute to sustained elevation of Dia level in response to receptor stimulation. Dia could activiate the protein kinase C (PKC) which appears to be a key enzyme in signal transduction, therefore, Dia is an important second messenger that has been implicated in the regulation of cell proliferation and many other cellular events[8]. We previously reported an increased intracellular $[Ca^{2+}]_i$ in VSMC exposed to AGEP-BSA which was likely involved with Dia, IP3, and PKC activity. Recently, we have obtained direct evidence that PKC activation was induced by AGEP-BSA.

Our results were consistent with that the vitamin E prevented AGEP-BSA-induced generation of Dia in VSMC when the cells were exposed to elevated level of glucose[10]. Recently, it was reported that vitamin E could inhibit the thrombin-induced generation of Dia via activation of Dia kinase[11]. We inferred that the inhibition of AGEP-induced generation of Dia by vitamin E was also mediated by an increased activity of Dia kinase.

When BSA was glycosylated with increasing concentrations of glucose or incubation time, both modified content among BSA and formation of AGEP-BSA would be increased. As an effective inhibitor of AGEP formation, AG prevents a variety of diabetic complications in experimental animal models and is currently being investigated in diabetic clinical trials. Previous study has demonstrated that AG can inhibit any Amadori products formed to AGEP[12]. Our results showed that AG-treated AGEP-BSA markedly attenuate

Dia level in VSMC which might be attributed to reduction of irreversible AGEP formation, since reduced AFU of AGEP-BSA treated by AG suggested that the formation of AGEP was significantly decreased.

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糖基化终产物对培养大鼠主动脉平滑肌细胞二酰基甘油的影响

R965.2

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关键词　高级糖基化终产物；甘油二酯类；血管平滑肌；培养的细胞；动脉粥样硬化；信号传递；维生素 E；胍类；磷脂酸类；放射自显影术

目的：研究糖基化终产物(AGEP)对主动脉平滑肌细胞信号转导环节之一二酰基甘油(Dia)的影响和维生素 E、氨基胍对 Dia 水平的干预作用．**方法**：采用放射酶标记、薄层层析、放射自显影技术测定细胞中 Dia 含量．**结果**：AGEP-BSA 孵育的平滑肌细胞 Dia 水平在 15 s 和 10 min 出现两个峰值，对照组和 AGEP-BSA 50，100，200 mg·L^{-1}作用 SMC 的 Dia 水平分别为(220±15) pmol·L^{-1}，(328±15) pmol·L^{-1}，(662±33) pmol·L^{-1}和(940±43) pmol·L^{-1}．Dia 水平也随 AGEP-BSA 糖基化的程度和修饰 BSA 的糖浓度增加而增高．维生素 E 和氨基胍可明显减弱 AGEP 刺激平滑肌细胞 Dia 水平升高的效应．**结论**：AGEP-BSA 能升高培养的大鼠血管平滑肌细胞中的 Dia 水平，维生素 E 和氨基胍能明显抑制这一效应．

（责任编辑　刘俊娥）