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Regulatory mechanism of gallic acid against advanced glycation end products induced cardiac remodeling in experimental rats

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ABSTRACT

Advanced glycation end products (AGEs) play a major role in the development of cardiovascular disorders in diabetic patients. Recent studies evidenced the beneficial role of phytochemicals in reducing the risk of cardiovascular diseases. Hence the present study was framed to investigate the protective role of Gallic acid (GA) on AGEs induced cardiac fibrosis. Rats were infused with *in vitro* prepared AGEs (50 mg/kg BW-intravenous injection) for 30 days. Further, GA (25 mg/kg BW) was administered to rats along with AGEs. On infusion of AGEs, induction of fibrotic markers, collagen deposition, oxidative marker NADPH oxidase (NOX-p47 phox subunit), AGE receptor (RAGE) and cytokines expression was evaluated in the heart tissues using RT-PCR, Western blot and immunostaining methods. AGEs infusion significantly ($P < 0.01$) increased the HW/BW ratio and fibrosis (4-fold) with increased expression of matrix genes MMP-2 and -9 ($P < 0.01$, respectively) in the heart tissues. Whereas, administration of GA along with AGEs infusion prevented the fibrosis induced by AGEs. Further, GA treatment effectively prevented the AGEs mediated up-regulation of pro-fibrotic genes and ECM proteins such as TNF-α, TGF-β, MMP-2 and -9 expression. In addition, the increased expression of NOX ($P < 0.01$), RAGE ($P < 0.01$), NF-κB ($P < 0.01$) and ERK 1/2 on AGEs infusion were normalized by GA treatment. Thus the present study shows the protective effect of GA on the fibrotic response and cardiac remodeling process induced by advanced glycation end products from external sources.

1. Introduction

Advanced glycation end-products (AGEs), are complex heterogeneous end-products of non-enzymatic glycation reaction, believed to participate in the microvascular and macrovascular complications such as nephropathy, arteriosclerosis, retinopathy, neuropathy, and cataracts in diabetic pathogenesis [1,2]. In the early-stage of glycation, these AGEs are generated via a glycosylation reaction between the amino groups of the body proteins with the aldehyde group of physiological sugars to form a Schiff's base; in turn, the Schiff's base rearranges to form Amadori products [3,4]. This AGEs formation also occurs in aging, but it occurs at an accelerated rate in clinical and experimental diabetes. Physiologically, in hyperglycemic conditions, the elevated serum glucose level facilitates the formation of AGEs [5].

Deposition of advanced glycation end-products, AGEs, reportedly contributes to the development of age-related and diabetes-related complications through both direct chemical mediated pathways through covalent cross-link formation, as well as, through cell surface receptor-mediated pathways [6,7]. Interaction of AGEs with the receptor, receptor for advanced glycation end products (RAGE) initiates the various metabolic pathways. The intricate involvement of AGEs in cellular oxidative damage comes from the end products' ability to disturb the redox status through their interaction with the specific receptor RAGE [8].

Upon infusion of AGEs into healthy rodents, an induction of AGE–RAGE induced cytosolic reactive oxygen species production with mitochondrial permeability transition and deficiency of mitochondrial complex 1 was observed [9]. Comparatively, increased oxidative stress was also observed in experimental rats followed by infusion of AGEs, which mimics the chronic condition of diabetic complications [10]. AGEs, rather than oxidative stress, are identified as potent inducers of vascular dysfunction [11]. In addition, the association of endothelial dysfunction with expression of downstream mediator protein kinase 1 (PKG-1) on AGEs infusion, emphasizes the role of AGEs in vascular dysfunction [12]. *In vivo*, the developmental nephron deficit in rats was associated with increased susceptibility to a secondary renal injury with exacerbated renal accumulation and increased collagen associated fluorescence of AGEs was found in AGE infused animals [13]. Although several lines of evidence have shown the involvement

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of AGEs in the development of cardiac complications, reports of findings on the direct impact of AGEs in the activation of cardiac fibrotic signals are not yet clear.

Gallic acid (GA), a polyphenol, possesses strong antioxidant, anti-inflammatory, antimutagenic and anticancer activity. It is also known to potentiate several pharmacological and biochemical pathways. Reports evidenced the restoration of lipid peroxidation, lysosomal damage and myocardial damage induced by isoproterenol upon GA treatment [14]. Gallic acid exhibits cytotoxicity against cancer cells, without harming healthy cells [15]. It can be used to treat albuminuria and diabetes [16] and also used as an antioxidant, which helps to protect human cells against oxidative damage. Studies suggest that the binding of the gallate compounds to lipid membrane is a principle determining factor of its antioxidant property [17]. Recently, we reported the protective role of gallic acid in cardiac H9C2 cells [18]. These findings evidenced the direct and indirect protective effects of antioxidant on the cardiovascular system. Thus, in the present study, *in vivo* studies have been carried out to elucidate the effect of AGEs on extra cellular remodeling and cardiac fibrosis.

2. Materials and methods

2.1. Chemicals

Rat serum albumin (RSA), gallic acid (GA), 3,3′-diaminobenzidine tetrahydrochloride (DAB) and Propidium iodide (PI) was procured from Sigma Chemical Co. (St. Louis, MO, USA). Trizol reagent, RT-PCR kit, and HRP-conjugated secondary antibody were purchased from GeNei (Bangalore, India). Gene-specific primers were purchased from Ocimum Biosolutions Inc., Netherlands. Primary antibodies, goat polyclonal IgG RAGE, Rabbit polyclonal IgG MMP-2, 9, NOX (p47 phox subunit) and β-actin and secondary antibodies Rabbit anti-goat IgG-HRP, Goat anti-rabbit IgG-HRP and Goat anti-rabbit IgG-FITC conjugate were purchased from Santa Cruz Biotechnology (San Diego, USA). All other chemicals used were of reagent grade.

2.2. Animals

Male Wistar rats (120–140 g) were used throughout the study. The animals were purchased from Tamil Nadu Veterinary and Animal Sciences University (TANUVAS), Madhavaram, Chennai, India. All animals were fed standard pellet diet (Gold Mohr rat feed, M/s. Hindustan Lever Ltd., Mumbai) and water *ad libitum*. They were maintained in a controlled environmental condition of temperature and humidity on alternatively 12 h light/dark cycles. The protocol of the experiment was approved by our institutional animal ethical Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India (IAEC No. 01/070/09).

2.3. Preparation and identification of AGEs

Soluble glycated rat serum albumin (AGE-RSA) was synthesized *in vitro*, according to the method described previously with modifications [19]. Solutions of D-glucose (1.6 M) and Rat serum albumin (100 mg/ml) have been co-incubated in sterile 10 mM phosphate buffer saline (PBS) was filtered through 0.2 μm filter and kept in dark for 6–8 weeks at 37 °C. Simultaneously, without glucose RSA alone incubated at the same condition served as control. After incubation, the free sugar was removed by extensive dialysis against 10 mM PBS, pH 7.4 for 48 h. The protein concentration was determined through the Bradford reagent.

2.4. Experimental design

In vivo, Wistar rats were randomly divided into four groups of ten animals in each group. Group I serves as normal control received vehicle saline; Group II animals were treated with gallic acid (GA) by oral gavage daily at a dose of 25 mg/kg BW/day for 30 days; Group III animals were infused with AGEs (50 mg/kg BW/day, i.v. – 30 days) [20] and Group IV animals treated with GA along with infusion of AGEs. At the end of experiment, the animals were killed and the heart tissue was excised immediately, snap frozen and kept at −80 °C for RT-PCR analysis or homogenized in 0.1 M Tris buffer pH 7.4 at 4 °C for other analysis of the study. A portion of left ventricular tissues were fixed in 4% paraformaldehyde solution and embedded in paraffin wax for immunohistochemical analysis.

2.5. Masson's trichrome staining

Paraffin embedded tissues sectioned at 5 μm thickness was used for collagen staining using Masson's trichrome. Briefly, the sections were de-paraffinised, rehydrated using series of alcohol and finally with distilled water. After that, the sections were stained in Weigert's iron hematoxylin solution and Biebrich scarlet acid fuschin solution. The slides were then treated with phosphomolybdic–phosphotungstic acid solution for 15 min after that, sections were transferred directly (without rinse) to aniline blue solutions for 5–10 min and were rinsed in distilled water and differentiated in 1% acetic acid solutions for 2–5 min. The sections were again rinsed in water and dehydrated very quickly through series of alcohol and mounted with DPX mounting medium. The ratio of interstitial fibrosis to the total left ventricular area was calculated from 20 randomly selected microscopic fields in five individual sections per heart using ImagePro Plus image analysis software (Media Cybernetics, Inc., Silver Spring, MD, USA).

2.6. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from the left ventricular heart tissues and cardiac cells of control and experimental groups using TRIzol reagent according to the manufacturer's protocol. To remove genomic DNA contamination, RNA samples were treated with RNase free DNase I (1 unit/μg RNA) at 37 °C for 30 min. The RNA integrity was confirmed by visualization of distinct 28 S and 18 S bands after electrophoresis on 2% agarose gel. RT-PCR was performed with GeNei M-MuLV RT-PCR kit with gene-specific primers. The PCR reactions were performed using gene specific primers listed in Table 1. The expressions were mRNA was normalized with GAPDH as internal control. The intensity of the bands visualized in 2% agarose gel was quantified using image density analysis software.

2.7. Western blot analysis

Tissue homogenate proteins (50 μg) was mixed with equal amount of sample loading buffer and separated under reducing condition using 12% SDS–PAGE. The separated proteins were transferred at 100 volts to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 1× Tris-buffered saline-Tween 20 (TBST), pH 7.5, containing 5% BSA for 45 min at room temperature and incubated at 4 °C overnight with specific antibodies to MMP-2, RAGE, NOX, TNF-α, NF-κB, ERK 1/2 and β-actin in 1× TBST containing 3% skimmed milk powder. After three washes with 1× TBST for 5 min each, the membrane was incubated for 1 h in HRP-conjugated anti-rabbit antibody at a dilution of 1:5000, washed three times with 1× TBST. Protein antibody complexes were detected by the addition of substrate diaminobenzidine.