Up-regulation of NO Production Induced by Maillard Product Derivatives and Activities of Natural Extracts Compounds Inhibiting NO Production in Murine Neuronal Cells

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Abstract

There has been growing evidence that Maillard reaction by-products, advanced glycation ends (AGE), causing renal and neuronal cells impairment and play a role in aging due to constant oxidative stress and inflammation. To indicate oxidative stress inside cells, nitric oxide is a well established marker despite its simplicity. Using high content assay of Murine neuroblastoma Neuro2A (N2A) cell line, it is possible to determine up-regulation of nitric oxide production as a result of AGE deposits as well as assessing potential drugs that may ameliorate this process. This paper is aimed to evaluate up-regulation of nitric oxide production from Neuro2A cells as induced by AGE made of chicken egg albumin (AGE-CEA) and bovine serum albumin (AGE-BSA).

$AGE\text{-}CEA_1$ (112µg/mL) induced production of NO as much as 11-13µM equivalent to $NaNO_2$. The increase of NO production was three to five fold compared to non-activated cells. It could also be concluded that AGE-CEA induced a stronger NO response than AGE-BSA. Activation of cells with $AGE\text{-}CEA_1$ (112µg/mL) for 24hours induced retraction and destruction of neurons, leading to a typical stressed cell shape. Natural extract from plants, apigenin, diosmetin, and naringenin, were dose-dependently able to down-regulate nitrite production in N2A cells.

Keywords: advanced glycation ends, nitric oxide, oxidative stress, Neuro2A

Introduction

Protein glycation naturally occurred in vivo during normal metabolism and had been particularly observed in platelet cells and neurons. It starts from reactions between reducing sugars and proteins to form Schiff base that continues to create intermediate products called Amadori, and later on become stable as the so-called advanced glycation end-products (AGEs) (Valencia et al, 2004). Apart formed inside cells, AGE was also obtained from food as a form of Maillard reaction derivatives (Glenn and Stitt, 2009). AGE content is proportional to the length of time and temperature used in cooking process.

Inside cells, nitric oxide (NO) means intercellular communication and has been regarded as a marker on oxidative stress conditions as well as an early indicator of inflammatory events (Khazei et al, 2008; Heneka, 2006). Murine neuroblastoma cells, Neuro2A (N2A), are known to be able to produce NO through enzymatic synthesis, through independent pathway (nNOS) (Lopez-Figueroa et al, 2002). Inducible pathway (iNOS) has been rarely reported in Neuro2A cell line, but could

increase extracellular NO concentration up to three folds upon bacterial lipo-polysaccharides (LPS) activation (Lindegren et al, 2003).
Apigenin, Naringenin, Diosmetin are purified natural extracts from plants that has potential to be developed as a health supplement. These compounds have been proven to reduce inflammatory activity through down-regulation of pro-inflammatory cytokines and NO on immune cells (macrophage) (Chandler et al, 2010).
The first objective of this research is to determine the production of NO in conditions which are activated by AGE produced from sugar (glucose) and chicken egg albumin (AGE-CEA) and bovine albumin (AGE-BSA). As a positive control, a combination of LPS and interferon-gamma (IFN-γ) is used and negative control is non-activated cells. The second objective is to measure the rate of decrease in NO production in Neuro2A cells which are activated as a result of addition of potentially inflammatory protective compounds namely apigenin, naringenin, and diosmetin.

Material and Methods

Medium

Culture medium used was DMEM (Gibco) enriched with 5% (v/v) FBS (Invitrogen), 1% (v/v) L-glutamine (Invitrogen), and 1% (v/v) streptomycin-penicillin (Invitrogen). All N2A cells were less than 15 passages.

Production AGE

AGE-BSA was produced from 1mg/mL of Bouvine serum albumin (BSA) and 100mM of D-glucose, 5 mM of D-fructose and 2mM of methylglyoxal. AGE- CEA1 produced from 1mg/mL of chicken egg Albumin and 100mM of D-glucose. AGE-CEA2 produced in the same way with AGE-CEA1, but with the addition of 5 mM of D-fructose and 2mM of methylglyoxal. All recipes of AGE were incubated for 37days at 60°C and dialysed for 48hours at a temperature of 8°C. Following that, AGE was sterile filtered into 1mL vials and stored at a temperature of -80°C. Before use, stock AGE dissolved in culture medium with a ratio of 1:10.

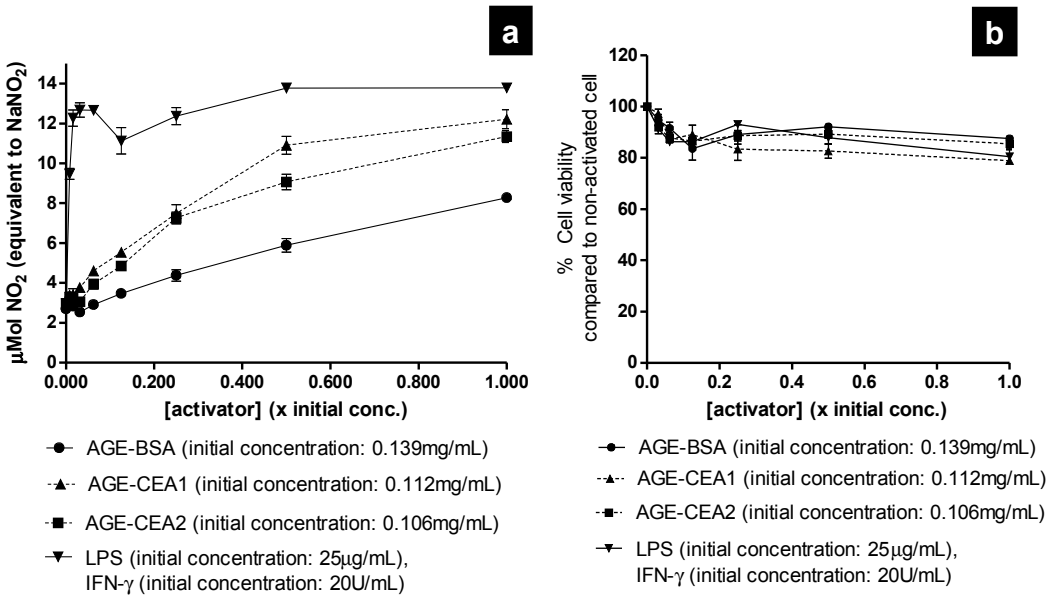
Inoculation

N2A cells were inoculated at 60,000cells/100µL on 96-well plate and were incubated at 37°C, 5% CO_2 for 24hours. Supernatant was removed and replaced with 100µL of AGE with different levels of concentrations (serially diluted 1:2), followed by incubation at 37°C, 5% CO_2 for 24hours. For the purpose of observation under a fluorescent microscope, N2A at 60,000cells/mL DMEM were inoculated on 6-well plate and all other treatments were same.

Observation

Griess method was employed to measure NO production, where absorbance was set to 540nm of wavelength. Concentration of nitrite was calculated based on the standard curve generated from the step-wise dilutions of sodium nitrite. Morphological observations was carried out with the aid of a fluorescent inverted microscope at a magnification of 200-400 times.

Result



Picture 3. (a) Nitrite production and (b) cell viabilities upon AGE and a combination of 25µg/mL of LPS and 20U/mL of IFN IFN-γ (positive control) activations on 60,000 cells/100µL N2A after being incubated for 24hours at 37°C dan 5% CO_2.

Conclusion

Maillard derivatives of chicken egg albumin and bovine serum albumin were able to induce up-regulation of nitric oxide production in N2A cell line. Further, AGE-CEA without methyl-glyoxal produced better activation in comparison to other recipes, but less potent than a combination of LPS and IFN-γ. Neurites were retracted and morphological changes were observed during activation. However, neurons were able to survive and restore its synaptic connections after two days. Apigenin, diosmetin, and naringenin were dose-dependently able to down-regulate nitrite production in N2A cells. This research also confirmed that N2A cell line has inducible pathway (iNOS).

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