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Short report

Various cellular stress components change as the rat ages: An insight into the putative overall age-related cellular stress network

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ABSTRACT

Cellular stress is mainly comprised of oxidative, nitrosative, and endoplasmic reticulum stresses and has long been correlated to the ageing process. Surprisingly, the age-related difference among the various components in each independent stress pathway and the possible significance of these components in relation to the overall cellular stress network remain to be clearly elucidated. In this study, we obtained blood from ageing rats upon reaching 20-, 40-, and 72-wk.-old. Subsequently, we measured representative cellular stress-linked biomolecules (H_2O_2, glutathione reductase, heme, NADPH, NADP, nitric oxide, GADD153) and cell signals [substance P (SP), free fatty acid, calcium, NF-κB] in either or both blood serum and cytosol. Subsequently, network analysis of the overall cellular stress network was performed. Our results show that there are changes affecting stress-linked biomolecules and cell signals as the rat ages. Additionally, based on our network analysis data, we postulate that NADPH, H_2O_2, GADD153, and SP are the key components and the interactions between these components are central to the overall age-related cellular stress network in the rat blood. Thus, we propose that the main pathway affecting the overall age-related cellular stress network in the rat blood would entail NADPH-related oxidative stress (involving H_2O_2) triggering GADD153 activation leading to SP induction which in-turn affects other cell signals.

1. Introduction

Cellular stress has been defined as a variety of processes that could trigger either an acute or chronic shift in the normal cellular homeostasis which in-turn would feature stress as an imposition of qualitative and/or quantitative loads to physiological homeostatic pathways (Barouki and Sitia, 2007). There are various types of cellular stresses occurring in a cell, among them: oxidative stress (OS), nitrosative stress (NS), and endoplasmic reticulum stress (ERS). OS represents an imbalance between pro-oxidant and anti-oxidant activities (Valko et al., 2007; Valko et al., 2006). NS is caused by the reaction of NO with oxygen or superoxide resulting in the formation of reactive nitrogen species with varying reactivity that could directly affect cellular enzyme activity (Fang, 2004; Valko et al., 2007). ERS is considered a cellular response activated when unfolded proteins accumulate within the ER in order to preserve ER function (Schonthal, 2012). All three independent stress pathways are composed of biomolecules that could likewise affect cell signalling and have previously been associated to the ageing process (Sverdlov et al., 2014) highlighting the importance of cellular stress.

Surprisingly, the age-related difference among the various components in each independent stress pathway and the possible significance of these components in relation to the overall cellular stress network remain to be clearly elucidated. A better understanding of the age-related cellular stress components with regard to the ageing process and in relation to the overall cellular stress network could shed light on the role of each cellular stress component in ageing-related diseases and, likewise, may lead to therapeutic strategies that would target these components.

2. Materials and methods

2.1. Animal handling

Throughout this study, we obtained 10 wk.-old Sprague-Dawley male rats ($n = 5$) (Nippon CLRA, Shizuoka, Japan) and extracted blood from ageing rats upon reaching 20- (20w), 40- (40w), and 72-wk.-old (72w). All rats were handled as previously described (Cueno et al., 2014; Cueno et al., 2013) and in accordance with the Nihon University guidelines (AP10D023) for animal studies. Rats were housed in individual stainless steel cages which were subsequently placed in a room under controlled conditions [temperature (23–25 °C), relative humidity

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(40–60%), and lighting (12h)] in order to acclimatize the rats and standardize the living conditions. Additionally, rats consumed 20–50 mL water and 2–5 briquettes daily which are both readily accessible. Heart blood was collected using a 25G × 1" needle 24 h post-injection and while rats were under an intraperitoneal anesthesia (Pentobarbital sodium).

2.2. Sample processing

Blood serum and cytosol were isolated and used for further downstream analyses. Blood serum isolation was performed using low-speed centrifugation. Briefly, heart blood (in 100 μL aliquots) was centrifuged at 500 × g for 30 min at 4 °C and the supernatant (blood serum) was transferred to a new tube. The precipitate (whole blood cell) was processed using the Cytosol/Particulate Rapid Separation Kit (BioVision Inc., California, USA) and, subsequently, Pierce® Detergent Removal Spin Columns (Thermo Scientific) was used to remove traces of detergents. For both processed blood samples, Pierce® Microplate BCA Protein Assay Kit-Reducing Agent Compatible Kit (Thermo Scientific, California, USA) was used to standardize the protein concentration. All kits used were according to manufacturer's recommendation.

2.3. Quantification of stress-linked biomolecules

Cellular stress-related biochemical components measured in the blood cytosol includes: total heme, hydrogen peroxide (H_2O_2), glutathione reductase (GR), NADP and NADPH, nitric oxide (NO), and GADD153. For OS, QuantiChrom™ Heme Assay Kit (BioAssay Systems, California, USA) was used to measure total heme levels (free heme and heme-proteins), Red Hydrogen Peroxide Assay Kit (Enzo Life Sciences, Pennsylvania, USA) was used to measure H_2O_2 amounts, Glutathione Reductase Activity Colorimetric Assay Kit (BioVision) was used to determine GR activity, and $NADP^+$/NADPH Assay Kit (BioAssay Systems) was used to determine NADPH and NADP levels. For NOS, QuantiChrom™ Heme Assay Kit (BioAssay Systems, California, USA) was used to measure cytosolic NO levels. All kits were used according to manufacturer's recommendation.

For ERS, GADD153 levels in the blood cytosol were quantified through ELISA. Briefly, antigen and sodium bicarbonate-sodium carbonate buffer (Polysciences, Inc., Taipei, Taiwan) solution was used for coating antigen (at 1 μg mL^{-1} concentration) on polystyrene plates. Overnight blocking was performed using PBS with 1% BSA blocking buffer (GeneTex Inc., Texas, USA). Moreover, recombinant GADD153 protein (GeneTex Inc.) was used to establish a protein standard while HRP-conjugated GADD153/CHOP antibody (Novus Biologicals, Colorado, USA) was utilized to detect GADD153. Peroxidase detection was done using SIGMA*FAST*™ OPD tablets (Sigma-Aldrich Co., Missouri, USA). Throughout the whole ELISA procedure, washing in-between steps was done using the PBS/Tween® Solution (AppliChem GmbH, Darmstadt, Germany), hydrochloric acid (1.0 M) was used as a stop solution, and measurements were done in Abs 450 nm.

2.4. Measurement of stress-linked cell signals

Biochemical components studied in both blood serum and cytosol are cellular stress-linked signals which include: free fatty acid (FFA), calcium, substance P (SP), and NF-κB (blood cytosol only). EnzyChrom™ Free Fatty Acid Assay Kit (BioAssay Systems) was used to quantify FFA levels. Calcium Colorimetric Assay Kit (BioVision) was used to measure blood cytosolic calcium amounts. NF-κB amounts were measured using NF-κB/p65 ActiveELISA™ Kit (Novus Biologicals). All kits were used according to manufacturer's recommendation.

For SP measurement, ELISA was performed as earlier described. Recombinant SP protein (Novus Biologicals) was used for protein standardization while SP antibody (GeneTex Inc.) was used as primary antibody for SP protein detection.

2.5. Network analysis of the overall cellular stress-linked components

The cornerstone of systems biology is network analysis and there are several computer-based modelling environments that have been developed to assist in understanding the network of various biochemical components involved in either cellular process or disease induction (Gilman and Arkin, 2002). Cytoscape is one such modelling environment that allows the user to combine biomolecular interaction networks into a single comprehendible conceptual framework (Shannon et al., 2003). We used Cytoscape to generate the probable cellular stress network structure based on the measured amounts and activity pattern of each stress-related component studied as the rat ages (20w, 40w, 72w). Network analyses of the different biochemical components and cell signals quantified in this study was subsequently performed. Among the various algorithms available for network analysis, we focused on the following topological centralities: (1) stress centrality to determine how involved a component is in the network; (2) betweenness centrality to establish whether a component is crucial in maintaining functionality and coherence; (3) closeness centrality to elucidate whether a component is functionally relevant to other components; (4) radiality centrality to highlight the possibility of a component to be relevant or irrelevant; (5) eccentricity centrality to emphasize the easiness of a component to be reached by other components; and (6) edge betweenness centrality to determine the degree of connection between two components (Koschutzki and Schreiber, 2008). Briefly, we first established the threshold for each centrality and compared these values to each biochemical component. For this study, we identified the degree of centrality of each biochemical component based on whether the biochemical component values were higher than the threshold.

2.6. Statistical analyses

Statistical analyses among the 20w, 40w, and 72w rat samples (blood serum and cytosol) were performed from the various biochemical data analyses made throughout the study. We first used the Andersen-Darling normality test to check whether the values obtained were normalized and, if $p > 0.05$ (considered acceptable), the statistical significance of differences were further elucidated by Tukey's test, wherein, a significance level of 95% ($p < 0.05$) was considered statistically significant.

2.7. Statement on reproducibility

All assays were conducted in duplicate in order to establish results reproducibility. Correctness of results obtained were confirmed through consilience in line with inferential reproducibility (Casadevall and Fang, 2010; Goodman et al., 2016).

3. Results and discussion

In general, we measured varying stress-linked components (biomolecules and cell signals) in the blood serum and cytosol at three different time points (20w, 40w, 72w) from an ageing rat. Throughout this study, 20w rats served as the control to which the other time points are compared to.

3.1. Cellular stress-linked biomolecules vary in the ageing rat blood

To establish blood cytosolic heme amounts, we measured total heme among the collected rat blood. We established that 40w and 72w rats have decreased and increased heme amounts, respectively (Fig. 1A). Heme is a biomolecule found to be involved in various biological reactions and, moreover, free heme and heme-proteins have been associated to OS induction (Balla et al., 2000; Hasan and Schafer, 2008). In this regard, we suspect that our observed changes in heme amounts are