Characterization of Human Serum Immunoglobulin G Modified with Singlet Oxygen

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Abstract Reactive oxygen species, as singlet oxygen ($^1\text{O}_2$), is continuously being generated by aerobic organisms, and react actively with biomolecules. At excessive amounts, $^1\text{O}_2$ induces oxidative stress and shows carcinogenic and toxic effects due to oxidation of lipids, proteins and nucleic acids. In our study, immunoglobulin G (IgG) was modified by $^1\text{O}_2$ generated by the ultraviolet (UV) irradiation of methylene blue. The modified IgG was characterized by UV spectroscopy, carbonyl content determination, thermal denaturation and electrophoretic study. Oxidation induced by modification of IgG by $^1\text{O}_2$ also analyzed by scavenging studies. It was found that ultraviolet absorption spectra of modified IgG shows marked hyperchromicity. The carbonyl content was found to be high in modified IgG as compared to native IgG which confirms its oxidation. Thermal denaturation of modified protein sample shows decrease in T_m value by 3 °C and less intensity banding pattern on polyacrylamide gel electrophoresis. The quenching effect of sodium azide provides clue for modification of IgG by methylene blue, as it is known $^1\text{O}_2$ scavenger. Hence, the IgG modified with $^1\text{O}_2$ may be one of the etiological pathogenic factors for rheumatoid arthritis and diabetes.

Keywords IgG · ROS · Singlet oxygen · Methylene blue · Sodium azide

Introduction

Molecular oxygen is intimately involved in living processes, although it has been shown to behave as a potential toxicant to all forms of life. This molecule may produce reactive derivatives (known as reactive oxygen species-ROS) as a result of energy absorption or electron transfer at many events in the cells, including as byproduct escapes from metabolic reactions, respiration or photosynthesis, or as part of organisms' defenses against pathogens [1, 2]. Singlet oxygen ($^1\text{O}_2$), the electronically excited state of molecular oxygen, is one of its more reactive and toxic forms. The activation of molecular oxygen to that energetic state with anti-parallel spin (singlet state) requires overcoming of spin restriction. The first excited singlet state, 1Dg , has two electrons with opposite spins in the same p^* orbital. The next higher excited singlet state, 1Sg , has one electron in each degenerated p^* orbital with opposite spins. Compared to the ground state of molecular oxygen (triplet), the 1Dg state is extremely reactive, and compared to the other electronically excited states, 1Dg state has a long half-life (of the order of 1 ms in water) to react chemically. The long half-life makes this excited molecule highly relevant in the biological environment [1, 3]. Therefore, the term “singlet oxygen” will be used in this paper referring exclusively to molecular oxygen in the 1Dg state. Singlet oxygen has a non-radical and electrophilic character. Thus, $^1\text{O}_2$ can induce oxidative reactions with organic compounds in its electron-rich moieties without the participation of free radicals. The high reactivity of $^1\text{O}_2$ with biological macromolecules makes it a potential aggressor when produced within the cell. Cell

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damage by free radicals appears to be a major contributor to aging and to degenerative diseases such as cancer, cardiovascular disease, cataract and brain dysfunction [4].

Immunoglobulin G (IgG) is the most abundant immunoglobulin constituting 75 % of serum immunoglobulins in human. IgG is a major protein of serum which is responsible for the interaction and clearance of antigens. Many studies show the presence of elevated levels of oxidized IgG in patient with various diseases such as rheumatoid arthritis, diabetes mellitus, systemic lupus erythematosus [4]. It is well known that IgG is quite vulnerable to ROS [5–7]. Many studies have shown the presence of elevated levels of oxidized IgG in patients with rheumatic diseases, such as RA [8–10]. It is also well documented that IgG behaves not only as an antibody, but also as a putative antigen for rheumatoid factor [9, 11]. Therefore, IgG is continuously exposed to oxidative stress, resulting in alterations in its conformation and function leading to modification of its biological properties.

homogeneity of isolated IgG was checked by polyacrylamide gel electrophoresis.

Modification of IgG

IgG (50 µg) was treated with various concentrations of methylene blue (25, 50, 100, 200 µM) and irradiated with UV radiation in PBS, pH 7.4. It was then dialyzed against PBS, pH 7.4.

UV Spectroscopic Studies

UV spectra were recorded on Shimadzu UV-240 spectrophotometer. The UV spectra were taken between 200–400 nm with a cell of 1 cm path length. Hyperchromicity was calculated by using the following equation [14]:

$$\% \text{Hyperchromicity} = (\text{Absorbance of modified sample} - \text{Absorbance of native sample}) / \text{Absorbance of modified sample} \times 100$$

Materials and Methods

PAGE electrophoretic unit and Rocker shaker 100 were from Bangalore Genei Pvt Ltd, India. Eppendorf centrifuge 5415R was from Germany. UV-1700 spectrophotometer was from Shimadzu Co. Japan. Gel documenting system was from Uvitech, India. Protein-A agarose affinity column from Genei India. All other chemical used in protocol were of analytic grade.

Purification of Immunoglobulin G

IgG from normal human sera was isolated by affinity chromatography on protein-Agarose CL-4B affinity column [12]. Serum (0.3 ml) diluted with equal volume of phosphate buffered saline (PBS), pH 7.4 was applied to the column equilibrated with the same buffer. Unbound IgG was removed by extensive washing with PBS, pH 7.4. The bound IgG was eluted with 0.58 % acetic acid in 0.85 % sodium chloride and neutralized with 1 ml of 1 M Tris-HCl, pH 8.5. 3.0 ml fractions were collected and read at 280 nm. The IgG concentration was determined by $1.40 \text{ OD}_{280} = 1.0 \text{ mg IgG/ml}$ and also by Lowry et al. method [13]. The isolated IgG was then dialyzed against PBS, pH 7.4 and stored at -20°C with 0.1 % sodium azide. The

Determination of Carbonyl Formation

Carbonyl contents of the native, irradiated and unirradiated IgG were analyzed by published procedures [15] with slight modification. The reaction mixture containing native, irradiated, and unirradiated protein (200 µl), 0.5 ml of 10 mM 2, 4-dinitrophenyl hydrazine (DNPH)/2.5 M HCl was added and thoroughly mixed. After addition of 250 µl TCA (20 %) and centrifugation, the pellet was collected and washed three times with 1 ml ethanol:ethyl acetate (1:1) mixture. The pellet was then dissolved in 1 ml of 6 M guanidine hydrochloride (GnHCl) solution and incubated at 37°C for 15 min. After centrifugation, the supernatant was collected and carbonyl content was estimated from the absorbance at 370 nm using a molar absorption coefficient $22,000 \text{ M}^{-1}\text{cm}^{-1}$. Samples were spectrophotometrically analyzed against a blank of 1 ml of 6 M GnHCl solution. Protein concentration was determined in the samples by the Lowry method [13]. Protein carbonyl content was expressed as nmol/mg.

Assay of Superoxide Radical

Superoxide radical was detected by Nakayama et al. [16]. A typical assay was performed in total volume of 3.0 ml

PBS pH 7.4, 33 mM NBT, 0.1 mM ethylenediamine tetraacetic acid (EDTA), and 0.06 % triton X-100 was started by addition of native IgG, unirradiated and irradiated IgG. The mixtures were measured spectrophotometrically at 560 nm against a blank which did not contain IgG samples.

Thermal Denaturation Studies

Thermal denaturation of native IgG, irradiated and unirradiated samples under identical conditions was evaluated by a temperature scan from 30 to 95 °C at an increment of 1 °C/min on a Shimadzu UV-240 spectrophotometer equipped with a temperature programmer and controller assembly. The change in absorbance at 260 nm was recorded and melting temperature (T_m) of the samples calculated [17].

Treatment of Modified IgG with Scavengers

IgG (50 µg/ml) was incubated with uric acid (50 mM), ascorbic acid (50 mM) and sodium azide (50 mM) for 2 h. After treatment for the specified time period, then methylene blue (100 µM) was added to the incubated mixture and final volume was adjusted to 3 ml with PBS (pH 7.5). The reaction mixture was then photoilluminated for 2 h. Absorbance was recorded at 280 nm by using UV spectrophotometer.

Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide gel electrophoresis (PAGE) of native and modified samples was performed as described [18]. Electrophoresis was done at 40 mA for 2 h at room temperature in Tris–acetate EDTA (TAE) buffer, pH 8.0. The bands so obtained were visualized after silver staining [19].

Results

Purification of Normal Immunoglobulin G and Modification

Immunoglobulin G was purified from normal human serum by Protein-A agarose CL-4B affinity column. The purified IgG from normal human sera was found to elute in a single symmetrical peak and gave a single band on 7.5 % PAGE (Fig. 1 inset). The affinity purified IgG were irradiated at room temperature in the presence of methylene blue using 254 nm UV light. The UV absorption of the irradiated IgG was recorded on Shimadzu UV spectrophotometer. The UV spectra of modified IgG revealed a marked hyperchromicity of 62.7 % at 280 nm (Fig. 1).

Electrophoresis of Immunoglobulin G modified with Methylene Blue

Native, modified and unirradiated IgG sample were electrophoresed on 7.5 % PAGE. The bands of modified and unirradiated IgG were diminished as compared to native IgG which might be due to some structural variation (Fig. 2).

Estimation of Carbonyl Contents in Modified IgG

The oxidation of protein typically results in an increase in carbonyl content, a known biomarker of oxidative stress. This is due to the oxidation of lysine, arginine, etc. residues in proteins. Therefore, oxidative involvement in modified IgG can be measured by estimation of carbonyl contents. After 2 h incubation, the carbonyl contents of native, unirradiated and irradiated IgG were found to be 11.2, 47.9, 55.1 nmol/mg protein, respectively. The modified IgG showed maximum carbonyl content (Fig. 3).

Fig. 1 Ultraviolet absorption spectra of native IgG (black line), irradiated IgG (green line) and unirradiated IgG (pink line)

Fig. 2 Polyacrylamide gel electrophoresis (PAGE) of native and modified IgG samples. IgG was loaded onto a 7.5 % polyacrylamide gel. Lanes 1 Native IgG, 2 unirradiated IgG with 100 µM methylene blue and 3 Irradiated IgG with 100 µM methylene blue

Thermal Denaturation

Thermal stability was also accessed using absorbance in UV region with increasing temperature. Native, irradiated and unirradiated samples were subjected to melting temperature between 30–90 °C. The process was characterized by determining the percent protein in denatured state as a function of increase in temperature by calculating T_m . The increase in absorbance at 280 nm with rise in temperature was recorded. The T_m of native IgG was found to be 77 °C and it decreases after modification with singlet oxygen. These results exhibit a net decrease of 3 °C in the T_m value for the irradiated IgG when compared to its native form. The decrease in T_m is due to structural and conformational

Fig. 3 Formation of carbonyl content by 50 µg/ml native IgG (black square), unirradiated IgG (pink square) and irradiated IgG (green square)

Fig. 4 Thermal melting profile of native IgG (black line), unirradiated IgG (pink line) and irradiated IgG (green line)

Fig. 5 The effect of various free radical scavengers, sodium azide (purple square), uric acid (yellow square) and ascorbic acid (blue square) on singlet oxygen-modified IgG (green square)

change in IgG which upon modification with singlet oxygen (Fig. 4).

Effect of Various Scavengers on Modified IgG

The scavenging effect of uric acid, sodium azide and vitamin C were analysed by incubating IgG with these scavengers and then modifying with methylene blue as earlier [20]. Sodium azide and to some extent uric acid showed the scavenging effect (Fig. 5).

Discussion

The oxidative modification of proteins, DNA and lipids has been implicated in the etiology of numerous disorders and diseases [21]. Oxidatively modified plasma protein can serve as an important in vivo biomarkers of oxidative stress. In the present study, human IgG was purified from normal human serum by affinity chromatography. The purified IgG was modified by singlet oxygen generated by the ultraviolet irradiation of methylene blue. It was observed that singlet oxygen caused structural distortion to the purified IgG as evident by a 62.7 % hyperchromicity at λ_{max} . The observed hyperchromicity is due to the exposure of aromatic amino acid or structural alteration in IgG upon singlet oxygen modification. The bands of less intensity on PAGE suggest that singlet oxygen modification resulted in extensive damage to IgG. Earlier studies have demonstrated that long-lived Tyr-derived peroxides are formed on proteins exposed to singlet oxygen and that these may promote damage to other targets via further free radical generation [22].

Protein carbonyl contents are actually the most general indicator and most commonly used markers of protein oxidation [15]. The recent studies on singlet oxygen-mediated protein oxidation and concentrates primarily on the mechanisms by which this excited state species brings

about changes to both the side-chains and backbone of amino acids, peptides, and proteins. Reactive peroxides may be important propagating species in protein oxidation as they can initiate further oxidation via both radical and non-radical reactions. Such processes can result in the transmittal of damage to other biological targets, and may play a significant role in bystander damage, or dark reactions, in systems where proteins are subjected to oxidation [21]. The carbonyl content of modified IgG is very high as compared to native IgG.

The production of superoxide radicals, via immune responses and normal metabolism, is a substantial contributor, if not the primary cause, of pathology associated with neurodegenerative diseases, ischemia reperfusion injury, atherosclerosis and aging [23–25]. The assays for generation of hydroxyl radical ($\text{OH}^\cdot$) and superoxide ($\text{O}_2^{\cdot-}$) were performed [26] to determine the presence of singlet oxygen. $\text{O}_2^{\cdot-}$ was detected by the reduction of NBT as described by published procedure [16]. The decrease in the absorbance at 560 nm confirms the absence of O_2 . Additional evidence for structural perturbations in IgG as a result of its modification with singlet oxygen was analyzed by melting profile. The melting curves of IgG after irradiation in the presence of methylene blue causes decrease in melting temperature (T_m). The decrease in T_m value to a extent of 3 °C showed that the unfolding of native IgG require high temperature as compared to irradiated IgG. The results indicated that stability of IgG decreases upon modification with methylene blue and require low temperature as compared to native IgG to melt. Scavenging studies were performed to analyze the effect of various scavengers on singlet oxygen generation. Among various scavenger used in our study, sodium azide was found to be the most effective scavenger. Earlier studies showed that long-lived peroxides are formed on free Tyr, Tyr residues in peptides and proteins, and model compounds on exposure to singlet oxygen generated by both photochemical and chemical methods. The yield of these species is significantly enhanced in D_2O and decreased by azide [22]. Sodium azide an efficient quencher of singlet oxygen [27] used frequently in qualitative diagnosis of singlet oxygen involvement in oxidation process. Thus, modification of IgG may produce neo-epitopes thus generated might play a role in the induction of circulating autoantibodies in rheumatoid arthritis.

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Conflict of Interest None.

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