Role of reactive oxygen species on the formation of the novel diagnostic marker ischaemia modified albumin

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MATERIALS AND METHODS

All reagents were obtained from Sigma-Aldrich (Poole, Dorset, UK) and normal human serum from the North London Transfusion Service (London, UK). Serum pH was 7.35–7.45 at 37°C and was unaltered by any of the reagents during the 15 minute experimental time course. Five millilitre serum aliquots (eight replicate experiments per group) were randomly selected for the following incubations for 15 minutes at 37°C. Group 1, peroxide treated (H₂O₂): serum was incubated with H₂O₂ 100 μM. Group 2, superoxide treated (O₂⁻): serum was incubated with a xanthine-xanthine oxidase O₂⁻ generating system consisting of 100 μM xanthine plus 0.05 U/ml xanthine oxidase (xanthine → urate⁻ + O₂⁻). Group 3, hydroxyl treated (·OH): ·OH was generated by the Fenton reaction and Cu²⁺ 0.1 mmol/l catalysed the generation of ·OH from H₂O₂ 100 μM (H₂O₂ + Cu²⁺ → ·OH + OH⁻ + Cu⁺). Group 4, Cu²⁺ control: aliquots of serum were incubated with 0.1 mM CuSO₄ as a technical control for group 3. Group 5, ·OH + MPG treated: aliquots of serum were incubated with the ·OH generating mixture (as in group 3) with the addition of MPG 1 mM. Group 6, control: serum was incubated for 15 minutes without the addition of any reagents.

Samples were withdrawn for analysis of IMA at baseline and after 2, 5, 10, and 15 minutes’ incubation. Samples were frozen at −70°C for blinded IMA determination by the ACB test (ACB Test, Ischemia Technologies Inc, Denver Colorado, USA) on a Roche cobas MIRA PLUS analyser (ABX Ltd, London, UK). The principle of the test is as follows. Co²⁺ is added to serum. Co²⁺ not sequestered at the N-terminus of albumin is detected by diithiothreitol as a colorimetric indicator. In normal serum, more Co²⁺ is sequestered at the N-terminus of albumin, leaving less Co²⁺ to react to form a coloured product. After chemical modification of serum, Co²⁺ is not sequestered at the N-terminus of albumin, leaving more free Co²⁺ to react. The total interassay coefficient of variation was 4.9–7.5% at 72.54–140.16 U/ml for quality control material. For human serum pools, the total coefficient of variation was 5.3–8.8% at 95.07–97.35 U/ml.

The percentage change in IMA in each group was calculated as follows: (IMA concentration at each time point − baseline IMA concentrations)/baseline IMA concentration × 100. Results are expressed as mean (SE) of eight replicate experiments. IMA concentrations for each treatment were time matched by repeated measures analysis of variance with Bonferroni post hoc comparisons by SPSS 11.0 statistical software (SPSS Inc, Chicago, Illinois, USA). Results were considered significant when p < 0.05.

RESULTS

Table 1 presents changes in IMA concentration. In control serum (group 6) IMA concentrations did not change significantly at any time point. Neither H₂O₂ (group 1) nor O₂⁻ (group 2) caused any significant change in IMA concentration during the experimental time course compared with control. However, generation of ·OH by the Fenton reaction (group 3) was associated with a rapid rise of IMA concentration. A maximum increase of 43.6% greater than baseline was observed at 15 minutes. The addition of the ·OH scavenger MPG (group 5) to the Fenton reaction mixture attenuated the production of IMA (no significant difference versus control group 6).

Separate titrimetric analysis confirmed that H₂O₂ concentration was reduced by 22.0 (3.0)% (four determinations) during 15 minutes’ incubation as a result of serum catalase activity. Serum albumin concentration, determined by immunonephelometric assay, was 42.4 g/l before treatment and was not altered by 15 minutes’ incubation with any of the reagents studied.

Since Cu²⁺ may interfere with the Co²⁺ binding assay, an important technical control in our experiments was group 4. We observed that Cu²⁺ 0.1 mM in the absence of ·OH generation resulted in no significant change in IMA concentrations. Theoretically, Cu²⁺ bound to the N-terminus prevents Co²⁺ binding because the binding constant for Cu²⁺ (K₉ = 1.5 × 10⁻⁶ M⁻¹) is many orders of magnitude higher than that for Co²⁺ (K₉ = 6.5 × 10⁻¹ M⁻¹). However, in these experiments we observed no appreciable interference of Cu²⁺.

DISCUSSION

In vivo modifications of the albumin N-terminus are proposed to be related to the ROS production during myocardial ischaemia–reperfusion. For example, an increase in IMA was observed in patients minutes after transient occlusion and reperfusion during coronary angioplasty. The

Abbreviations: ACB, albumin Co²⁺ binding; IMA, ischaemia modified albumin; MPG, mercaptopropionylglycine; ROS, reactive oxygen species; SOD, superoxide dismutase
present in vitro investigation shows for the first time that IMA formation is directly related to ROS generation and provides novel information on the nature of the species contributing to IMA formation, namely 'OH.

Previous studies in vitro have shown that the exposure of albumin to 'OH generated by copper (at similar concentrations to those used in our experimental series) and ascorbate resulted in modification of albumin in a site specific manner, rather than generalised degradation. 

On the other hand, NO is released during reperfusion, and can interact with the reactive oxygen species such as superoxide anion, forming the powerful oxidant ·ONOOH, which is responsible for biological damage. 

There have been several studies on the importance of NO in vascular biology and its potential role in physiopathology, and the importance of understanding its biochemistry in pathological conditions associated with NO deficiency is gaining increasing appreciation. 


Table 1 Ischaemia modified albumin percentage change from baseline concentrations

<table>
<thead>
<tr>
<th>Group</th>
<th>Incubation time (min)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>1: H_{2}O_{2}</td>
<td>-0.7 (1.0)</td>
<td>-7.1 (4.3)</td>
</tr>
<tr>
<td>2: O_{2}^{•}</td>
<td>3.9 (2.4)</td>
<td>5.5 (3.0)</td>
</tr>
<tr>
<td>3: 'OH</td>
<td>28.3 (3.4)</td>
<td>34.7 (3.6)</td>
</tr>
<tr>
<td>4: Cu^{2+} control</td>
<td>4.1 (1.4)</td>
<td>6.4 (0.7)</td>
</tr>
<tr>
<td>5: 'OH + MPG</td>
<td>6.2 (8.5)</td>
<td>6.1 (8.7)</td>
</tr>
<tr>
<td>6: control</td>
<td>0.7 (1.5)</td>
<td>-0.5 (0.8)</td>
</tr>
</tbody>
</table>

Data are expressed as mean (SEM) of eight replicate experiments. p Values relate to comparison with group 6 (control).

NS, not significant (repeated measures analysis of variance with Bonferroni post hoc test).