

SCIENTIFIC LETTER

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

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Ischaemia modified albumin (IMA) is a recently developed biomarker of transient myocardial ischaemia.¹ Circulating IMA is increased in patients with myocardial ischaemia, after percutaneous coronary intervention,^{2,3} or in acute coronary syndromes.⁴ The test has recently been licensed by the US Food and Drug Administration for diagnostic use in suspected myocardial ischaemia. IMA is serum albumin in which the N-terminus has been chemically modified. The diagnostic albumin Co²⁺ binding (ACB) test is based for IMA on the observation that the affinity of serum albumin for Co²⁺ is reduced after N-terminus modifications. It has been proposed that reactive oxygen species (ROS) such as superoxide (O₂⁻) and hydroxyl (OH) radicals generated during myocardial ischaemia–reperfusion modify the N-terminus of serum albumin resulting in IMA formation but, so far, direct evidence to support this is scarce. We hypothesised that ROS generation causes the formation of IMA. Our objective was to model the formation of IMA in vitro by using chemically generated ROS and the OH radical scavenger mercaptopropionylglycine (MPG).

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 reactants 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 + Cu⁺ → OH + OH⁻ + Cu²⁺). 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 reactants.

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 dithiothreitol 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 reactants 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⁺ (*K*_a = 1.5 × 10¹⁶ l/mol) is many orders of magnitude higher than that for Co²⁺ (*K*_a = 6.5 × 10³ l/mol). 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.^{2,3} The

Abbreviations: ACB, albumin Co²⁺ binding; IMA, ischaemia modified albumin; MPG, mercaptopropionylglycine; ROS, reactive oxygen species; SOD, superoxide dismutase

Table 1 Ischaemia modified albumin percentage change from baseline concentrations

| Group | Incubation time (min) | | | | p Value |
|----------------------------------|-----------------------|------------|------------|------------|---------|
| | 2 | 5 | 10 | 15 | |
| 1: H ₂ O ₂ | -0.7 (1.0) | -7.1 (4.3) | 2.5 (2.2) | 0.4 (2.6) | NS |
| 2: •O ₂ ⁻ | 3.9 (2.4) | 5.5 (3.0) | 0.1 (2.0) | -2.7 (2.8) | NS |
| 3: •OH | 28.3 (3.4) | 34.7 (3.6) | 38.2 (3.2) | 43.6 (6.5) | <0.0001 |
| 4: Cu ²⁺ control | 4.1 (1.4) | 6.4 (0.7) | 5.8 (1.6) | 4.0 (1.3) | NS |
| 5: •OH + MPG | 6.2 (8.5) | 6.1 (8.7) | 8.6 (7.4) | 6.0 (8.7) | NS |
| 6: control | 0.7 (1.5) | -0.5 (0.8) | 1.7 (1.7) | 1.4 (0.9) | NS |

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).

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.⁵ ROS generation in vitro caused structural changes in a synthetic N-terminus tetrapeptide, an octapeptide, and human albumin with loss of Co²⁺ binding capacity.⁶ Our data are in accordance with these findings, since we show here that •OH generation caused a rise of IMA concentrations. Moreover, the role of •OH was confirmed by attenuation of IMA formation in the presence of the •OH scavenger MPG.

Intriguingly, neither H₂O₂ nor •O₂⁻ alone resulted in significant IMA formation. It seems unlikely that the lack of effect of H₂O₂ on IMA formation was caused by inactivation by the enzyme catalase present in serum, since we observed only partial degradation of H₂O₂ (22%) during 15 minutes' incubation. Thus, the H₂O₂ concentration remained high (> 70 µM) throughout the experimental time course. The dismutation of •O₂⁻ by extracellular superoxide dismutase (SOD) present in serum undoubtedly occurs in vitro, just as it does in vivo. Moreover, the presence of intracellular SOD isoenzymes in vivo may further reduce the availability of •O₂⁻. At present, we cannot say whether the efficiency of extracellular SOD in accomplishing the dismutation of •O₂⁻ accounts for the lack of effect of •O₂ or whether •O₂⁻ has low chemical reactivity with albumin in vitro. It has been suggested that •O₂⁻ per se is not deleterious but serves as a source of highly reactive secondary species such as •OH, which are responsible for biological damage.

These data support the hypothesis that ROS, and specifically in our hands •OH, may chemically modify human serum albumin, resulting in IMA formation. This appears to be a plausible mechanism underlying this new diagnostic test. To improve the clinical utility of IMA a clear understanding of

the mechanism of the reaction relevant to in vivo ischaemia-reperfusion is essential. Clearly, further in vivo studies supported by data from clinical trials are required to confirm the novel mechanism we have identified in these in vitro studies. Moreover, it will be important to elucidate the anatomical sites of IMA formation and its value in the diagnosis of disease states associated with oxidative stress, including myocardial ischaemia.

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